THE EFFECT OF POTASSIUM ON THE WASHING AND DILUTION OF MAMMALIAN SPERMATOZOA

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The effect of washing ram, bull and rabbit spermatozoa once and twice has been studied by White (1953). It was found in these experiments that a single washing had no significant effect on motility but that washing twice could be deleterious. A decrease in total oxygen uptake and lactic acid production during the 5-hour observation period was also noted on washing, and evidence was obtained suggesting that the former was due largely to the removal of bacteria and the latter to the washing out of essential components of the glycolytic system from the spermatozoa.

Assuming that the reduced motility on repetitive washing was caused by leaching out intracellular material concerned with lactic acid production, it was thought that the adverse effect of washing mammalian spermatozoa might be prevented by including in the sodium phosphate-fructose diluent substances likely to be important in the glycolytic cycle. Experiments along these lines are reported here, in which it is shown that the adverse effect of repetitive washing on the motility of ram and bull spermatozoa is reduced by the addition of potassium to the diluent used for washing. The success of potassium in these experiments suggested that it might also be helpful in preventing the deleterious effect of dilution on the motility of mammalian spermatozoa (Salisbury, Beck, Cupps and Elliot, 1943; Emmens and Swyer, 1948; Cheng, Casida and Barrett, 1949) and investigations of the possibility are included in the paper.

Materials and Methods.

Bull and rabbit semen was collected by means of the artificial vagina, and ram semen by electrical stimulation as described by Gunn (1936). In all cases only apparently normal specimens of good motility were employed. Experiments with ram and rabbit semen were started immediately after collection and those with bull semen within 2 hours of collection—the semen being stored during this period at about 10° C.

Unwashed spermatozoa suspensions were prepared by diluting 1 ml. of semen with 9 ml. of diluent in a graduated centrifuge tube, and washed spermatozoa were prepared by spinning this suspension four times at 1,500 r.p.m. for 10 minutes, the supernatant being drawn off and replaced by diluent after each centrifuging and the spermatozoa redispersed by sucking up and down in a wide bore Pasteur pipette fitted with a rubber teat. Centrifuged, but

unwashed, spermatozoa suspensions were prepared by centrifuging the diluted (1 in 10) semen four times and redispersing the cells after each spinning without drawing off the supernatant.

In the dilution experiments counts of spermatozoa were made in duplicate with neat semen, using an improved Neubauer haemocytometer under high power. The semen was then serially diluted to give, with ram and bull spermatozoa, cell concentrations of 200, 20 and 2×10^6/ml. and with rabbit 20 and $0{\cdot}4 \times 10^6$/ml.

Spermatozoa suspensions were incubated in tubes at 37° C. for 3 hours in the case of the ram and bull and at room temperature for 4 hours with the rabbit. Small drops were removed at hourly intervals for the scoring of motility as introduced by Emmens (1947); full motility being scored as 4 and complete immotility as zero.

The isotonic diluents of pH = 7·0 had the following composition:

Sodium. 0·032 M–$NaH_2PO_4H_2O$, 0·048 M–$Na_2HPO_412H_2O$, 0·40 M–NaCl, 0·22 M–fructose.

Sodium-Potassium. 0·32 M–$NaH_2PO_4H_2O$, 0·048 M–$Na_2HPO_412H_2O$, 0·036 M–NaCl, 0·004 M–KCl, 0·022 M–fructose.

Sodium-Protein. 0·032–$NaH_2PO_4H_2O$, 0·048 M–Na_2 HPO_412H_2O, 0·040 M–NaCl, 0·022 M–fructose, 0·25 p.c. (W/V) crystalline plasma albumen (Armour).

All diluents were freshly prepared each day by the appropriate dilution of 0·4 M stock solutions of the AR salts with glass distilled water, solid fructose being added to give a concentration of 0·4 p.c. (W/V). Stock phosphate and chloride solutions were stored in a refrigerator and discarded at the first signs of mould growth. The pH of all diluents was checked with the glass electrode prior to use.

In the analyses of variance the total motility score has been used as unit observation and the interaction mean square as the error term.

RESULTS.

Although it was thought that the adverse effect of repetitive washing on the motility of spermatozoa (White, 1953) was caused by the removal of important intracellular substances, the possibility of mechanical damage due to centrifuging could not be excluded. Experiments to obtain more information on this point were therefore undertaken in which motility comparisons were made between unwashed, washed spermatozoa and those centrifuged without

TABLE 1.

Summary of the analyses of variance for the data of Figs. 1 and 2, showing the variance ratios, with the interaction (error) mean square in italics at the base of the columns.

Source of variation	Ram		Bull	
	D. of F.	Variance ratio	D. of F.	Variance ratio
Between treatments	2	489·1**	2	40·8**
Unwashed v. washed	1	725·6**	1	61·2**
Unwashed v. centrifuged	1	0·1	1	0·0
Between ejaculates	2	4·2	5	11·5**
Interaction (Error)	4	*1,215*	10	*18,640*

** $P < 0{\cdot}01$.

washing. Fig. 1 gives the results for three ram ejaculates using the sodium diluent and Fig. 2 the corresponding data for six bull ejaculates. The analyses of variance (Table 1) show highly significant variation between treatments for each species, and it is clear that this arises because of the significantly lower motility scores of the washed spermatozoa compared with either the unwashed or centrifuged cells. There was no significant difference in motility score between the unwashed and centrifuged groups in either species.

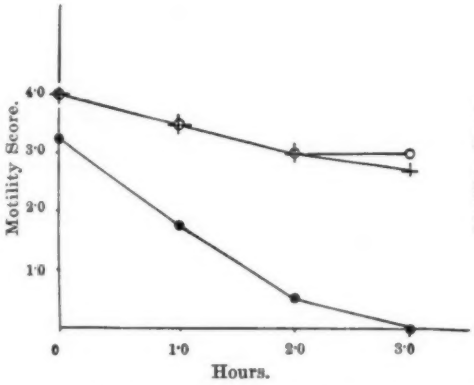

Fig. 1. The relative effect of washing and centrifuging four times in a sodium phosphate-fructose diluent on the motility of ram spermatozoa at 37° C. Each point represents the mean motility score of three ejaculates.

+——+ Unwashed.
O——O Centrifuged but unwashed.
●——● Washed.

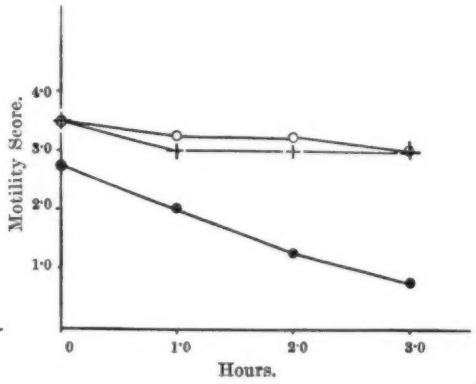

Fig. 2. The relative effect of washing and centrifuging four times in a sodium phosphate-fructose diluent on the motility of bull spermatozoa at 37° C. Each point represents the mean motility score of six ejaculates.

+——+ Unwashed.
O——O Centrifuged but unwashed.
●——● Washed.

Since damage by centrifuging was not an important factor in the washing effect, attempts were made to prevent it by supplementing the diluent with substances likely to be leached out of the spermatozoa, the guiding principle in the selection of such substances being a possible connection with the glycolytic system. Early experiments indicated the importance of potassium and it was found that the adverse effect of washing ram spermatozoa in the sodium diluent could be completely prevented by the inclusion of 0·004 M–KCl. Fig. 3 gives the results for six ram ejaculates and Table 2 the analysis of variance. A highly significant difference is seen between treatments, arising from the fact that the motility of both the unwashed and potassium-washed spermatozoa was much superior to the motility of those washed in the potassium-free diluent ($P<0·01$). No significant difference occurred between the unwashed and potassium-washed groups.

Similar, but less striking, results were obtained with six bull ejaculates (Fig. 4). The analysis of variance (Table 2) again shows highly significant treatment effects. As with the ram, the motility of bull spermatozoa washed in the potassium diluent was superior to that of spermatozoa washed in the potassium-free diluent, but a highly significant difference occurred between the unwashed and potassium-washed cells, indicating that potassium was only partly successful in preventing the washing effect in this species.

TABLE 2.

Summary of the analyses of variance for the data of Figs. 3 and 4, showing the variance ratios, with the interaction (error) mean square in italics at the base of the columns.

Source of variation	D. of F.	Variance ratios	
		Ram	Bull
Between treatments	2	51·1**	19·9**
Unwashed v. washed in sodium diluent	1	77·9**	39·8**
Unwashed v. washed in sodium-potassium diluent	1	0·0	11·8**
Between ejaculates	5	3·5*	7·5**
Interaction (Error)	10	*23,369*	*36,521*

* $P < 0.05$. ** $P < 0.01$.

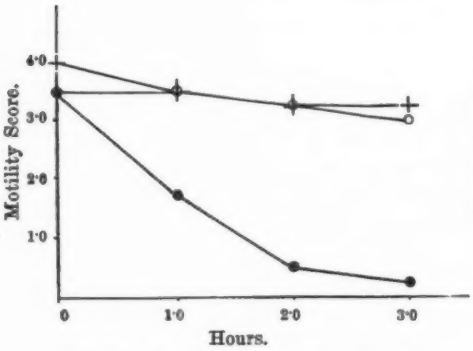

Fig. 3. The effect of washing four times in sodium and sodium-potassium diluents on the motility of ram spermatozoa at 37° C. Each point represents the mean of six ejaculates.

+——+ Unwashed in sodium.
O——O Washed in sodium-potassium.
●——● Washed in sodium.

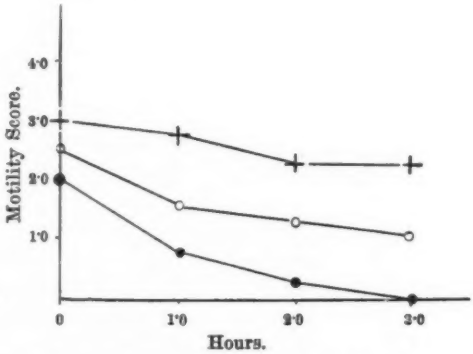

Fig. 4. The effect of washing four times in sodium and sodium-potassium diluents on the motility of bull spermatozoa at 37° C. Each point represents the mean of six ejaculates.

+——+ Unwashed in sodium.
O——O Washed in sodium-potassium.
●——● Washed in sodium.

The relationship between the exhaustive washing and dilution of spermatozoa has been discussed by Emmens and Swyer (1948) who regard them as parallel phenomena; it was of interest therefore, in view of its benefit in the washing experiments, to see if potassium would prevent the harmful effect of dilution on bull and ram spermatozoa. The motility scores were determined for ram and

bull spermatozoa incubated for 3 hours at 37° C at three dilutions (200, 20 and 2×10^6 cells/ml.) in the potassium and potassium-free diluents. Potassium had little or no effect on motility at the highest or lowest cell concentrations. However, with ram spermatozoa at a cell concentration of 20×10^6/ml. the analysis of variance (Table 3) shows that motility in the potassium diluent was significantly better than in the potassium-free diluent. The motility of bull spermatozoa at this concentration was always at least slightly superior in the potassium diluent but not significantly so (Table 3). This beneficial effect of potassium at cell concentration of 20×10^6/ml. is expressed graphically in Fig. 5.

TABLE 3.

Summary of the analyses of variance for the motility score of ram and bull spermatozoa at a concentration of 20 × 10⁶/ml. (Fig. 5). The interaction (error) mean square is shown in italics at the base of the columns.

Source of variation	D. of F.	Variance ratios	
		Ram	Bull
Between treatments	1	15·87*	2·94
Between ejaculates	5	4·62	2·30
Interaction (Error)	5	*27,177*	*57,469*

* $P < 0.05$.

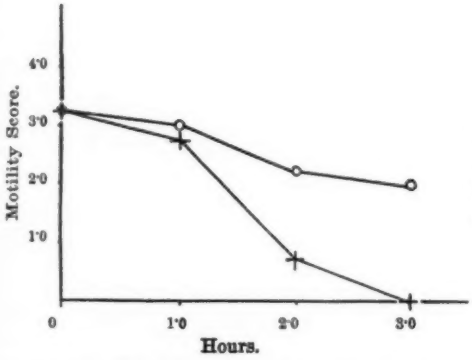

Fig. 5. The hourly motility score of ram spermatozoa incubated at a concentration of 20×10^6/ml. in sodium (+———+) and sodium-potassium (O———O) diluents at 37° C. Each point represents the mean motility score of six ejaculates.

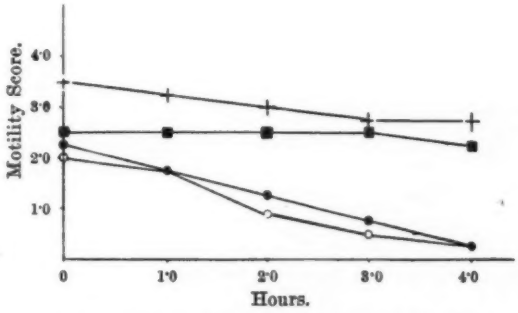

Fig. 6. The hourly motility score of rabbit spermatozoa during four hours at room temperature in sodium, sodium-potassium and sodium-protein diluents. Each point represents the mean of four ejaculates.

+———+ Sodium diluent (20×10^6 cells/ml.).
■———■ Sodium-protein diluent ($0{\cdot}4 \times 10^6$ cells/ml.).
●———● Sodium-potassium diluent ($0{\cdot}4 \times 10^6$ cells/ml.).
O———O Sodium diluent ($0{\cdot}4 \times 10^6$ cells/ml.).

The success of potassium in improving the motility of ram spermatozoa moderately diluted at 37° C. suggested that it might be important in the dilution effect studied by Emmens and Swyer (1948) using rabbit spermatozoa where, although dilution was more severe, the experiments were conducted at room temperature. These workers found that in this species the deleterious effect of dilution on motility could be partly prevented by a variety of high molecular weight substances, including starch and certain proteins. If potassium were found to be as effective as these agents in countering the dilution effect then it would be evidence that they might function by preventing the leakage of this element from the cells.

Fig. 6 gives the results of experiments designed to explore this possibility, in which the effect was observed of diluting rabbit spermatozoa 1 in 50 into sodium, sodium-potassium and sodium-protein diluents—the spermatozoa being suspended initially in the sodium diluent at a cell concentration of 20×10^6/ml. Analysis of variance showed that the adverse effect of dilution is not ameliorated by potassium under conditions where plasma albumen is beneficial.

DISCUSSION.

It would appear that the important substance lost from ram spermatozoa on repetitive washing is potassium since its inclusion in the diluent completely prevented the harmful effect of washing on motility (Fig. 3). With bull spermatozoa it is probable that other substances in addition to potassium are lost from the cell since potassium, although it had a significant effect, was not completely successful in maintaining motility at the level of the unwashed controls (Fig. 4). At all events these experiments establish the importance of potassium in the normal functioning of the spermatozoa of both species. Apparently the only reference in the literature to the effect of potassium on the motility of mammalian spermatozoa is that of Lardy and Phillips (1943) who, without giving any data, state that potassium in a concentration of 0·005 M is necessary for the maintenance of the full motility of bull spermatozoa.

The function of potassium in the spermatozoa is believed to be associated with the glycolytic cycle since it has been shown (White, 1953) that washing in a potassium-free diluent significantly decreases lactic acid production by ram and bull spermatozoa and that the addition of potassium to the diluent restores the ability to produce lactic acid (White, unpublished data). This is in keeping with the claim of Lardy and Zeigler (1945) that potassium catalyses the transfer of phosphate from 2-phosphopyruvic acid to adenosine diphosphate which is believed to be an important reaction in the glycolytic cycle.

Ludwig, Greig and Peterson (1951) have studied the loss of potassium from washed erythrocytes suspended in potassium-free diluents and claim that

it is reduced by acetyl choline and increased by physostigmine, suggesting that intracellular potassium levels are dependent upon choline esterase activity. It would be interesting to see if similar effects are obtained with washed spermatozoa, particularly as phosphoryl choline, a possible substrate for choline esterase, is known to be a constituent of mammalian semen (Lundquist, 1946).

According to Bernstein (1933) bull semen contains considerable quantities of potassium, so that little advantage might be expected from including potassium in a diluent for bull spermatozoa at high cell concentrations. This is in accordance with the observation that the motility of bull spermatozoa in the potassium diluent was no better than in the sodium diluent at a cell concentration of 200×10^6/ml. There are apparently no figures for the potassium content of ram semen in the literature, but it is obvious that, as with the bull, the potassium diluent is not superior at high concentrations of ram spermatozoa.

On moderate dilution (20×10^6 cells/ml.) however, it does seem, particularly with ram spermatozoa, that potassium is a limiting factor in the maintenance of motility (Fig. 5). This being so, and assuming a correlation between motility and capacity to fertilize, it would seem advisable that diluents for artificial insemination should contain at least 0·004 M potassium. It should be noted in this regard that many of the diluents at present used are either completely lacking in potassium or are deficient in the element.

At very high dilutions ($2{\cdot}0 \times 10^6$ cells/ml.) substances other than potassium must be limiting since motility in the potassium diluent is not, under these conditions, better than in the potassium-free diluent. The results shown in Fig. 6, together with the analysis of variance, would indicate that the beneficial effect of plasma albumen on rabbit spermatozoa at high dilutions is not related to the prevention of potassium loss from the cells.

SUMMARY.

In motility studies at 37° C. over 3 hours using ram and bull spermatozoa washed four times in a sodium phosphate-fructose diluent, it has been shown that the adverse effect of washing was not due to damage from centrifuging and that it could be prevented by the addition of 0·004 M–KCl to the diluent.

Similar observations on the motility of ram and bull spermatozoa at three dilutions (200, 20 and 2×10^6 cells/ml.) in potassium and potassium-free diluents indicated that at the intermediate cell concentration motility was superior in the potassium-containing diluent, but that at the higher and lower cell concentrations potassium had little or no effect.

Motility studies at room temperature over a period of 4 hours at cell concentrations of 20 and $0{\cdot}4 \times 10^6$/ml. showed that 0·004 M–KCl was not effective in preventing the deleterious effect of dilution on rabbit spermatozoa under conditions where 0·25 p.c. (W/V) plasma albumen was beneficial.

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FACTORS AFFECTING THE INTRAPLEURAL PRESSURE AND PULMONARY VENTILATION IN RATS[1]

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Investigations of the lymphatic absorption of material from the serous cavities have indicated that the rate of this absorption is influenced by the rate and force of the respiratory movements, particularly those of the diaphragm (cf. Florey, 1927; Higgins, Beaver and Lemon, 1930; Cooray, 1949; Courtice and Simmonds, 1949; Courtice and Steinbeck, 1950). The pressure changes occurring within the pleural cavity as a result of changes in respiratory movement probably play an important part in the processes underlying absorption by the lymphatics. The protein molecules or particles injected have first to pass through a serous membrane, then the membranous wall of the lymphatic capillary and finally must be propelled along the main lymph trunks passing through the mediastinum to enter the veins at the base of the neck.

Before investigating further some of the factors, in particular diaphragmatic movement, which might affect lymphatic absorption from the pleural and peritoneal cavities, it was decided to analyse the changes which these factors produce in the respiratory pattern, pulmonary ventilation and intrapleural pressure.

METHODS.

Albino rats of the Wistar strain were used. The high respiratory rate and small tidal air changes of the unanaesthetized rat necessitated a system of recording capable of estimating accurately rapid volume changes of small degree. Ordinary spirometric techniques involving the use of valves are not satisfactory at high respiratory rates and systems with a large dead space and high resistance are unsatisfactory for ventilation studies in small laboratory animals.

The method employed for determining the pulmonary ventilation and respiratory rate was a modification of that used by Guyton (1947). The system used is shown diagramatically in Fig. 1 and the actual arrangement of the various components of the apparatus is shown in Fig. 2. The principle of the method involves the recording and calibration of the pressure changes that occur when the animal breathes in a closed air system. With each inspiration a small amount of air equal to the animal's tidal volume is removed from the system reducing

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the pressure within the circuit. This reduction in pressure effects a movement in the diaphragm of the transducer altering its electrical capacity. These transduced barometric changes are then carried through a suitable amplification circuit to a recording unit. The tracings so produced record the respiratory patterns, rates and tidal air changes of the subject.

The rat was immobilized by suspending the animal supported in a cloth jacket designed to furnish a comfortable but efficient means of restraint when the animal was sewn into it. This seemed a more effective method of restraint than the glycerine coated tubes which McCutcheon (1951) used. The animals appeared quiet and reasonably content after an initial period of excitement had passed.

The respiration chamber was made of $\frac{3}{16}$-inch perspex and measured 3 inches cube. The hole to receive the rat's head was 1¼ inches diameter with six threaded brass studs spaced around its circumference to support the aluminium collars. The closed air system used was the same as that described by Guyton, the animal breathing fresh air passing through the respiration chamber at a constant rate of flow. Reservoir bottles used were of 4 litre capacity (Fig. 1A). The rat's head was sealed in the respiration chamber (Fig. 1B) using two thin dental dam sealing collars with holes of appropriate size. Small radial cuts were made at the edge of these holes to minimize constriction and petroleum jelly used as a sealing medium on the rat's neck and hair between the rubber collars. The thin rubber sealing collars were supported by two aluminium discs of similar size, with nonrestricting central holes. The composite collar was held by the threaded studs around the edge of the opening into the respiration chamber. A seal was completed between rat and chamber by attaching a thicker rubber sealing disc between the chamber and the aluminium collar and screwing the collar tightly against the chamber. When supported at an appropriate height and with collars screwed into position, the rat was free of any weight of the collars. Details of the chamber and of the collars are shown in Fig. 3.

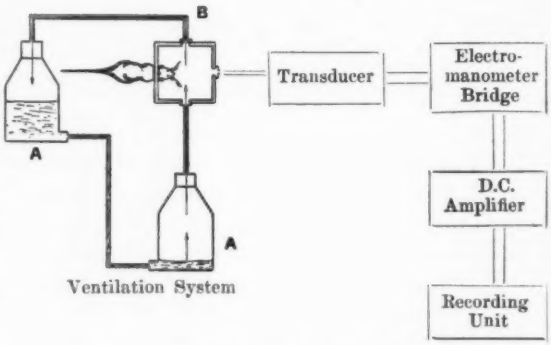

Fig. 1. Diagram of respirometer circuit. (AA) Air and water reservoirs. (B) Respirometer chamber.

All connecting tubes used in the system were of as large a diameter as was considered necessary to reduce the establishment of pressure equilibrium to a minimum. That this lag would be a factor in producing inaccuracies in our recordings is recognized but is not considered to invalidate the results recorded.

The respiration chamber and air systems connect through a lead tube to the recording system. A three-way cock allowed the system to be opened to the atmosphere when required. A Sanborn Electromanometer for which a more sensitive transducer was constructed was used for determining pressure variations. The apparatus consisted basically of a power supply and a balanced A.C. bridge. The transducer acted as the variable element in one leg of the bridge. The transducer was a capaciter having one fixed and one moving plate. The moving plate was in the form of a shallow cone-shaped tin-foil diaphragm. This diaphragm formed one side of an enclosed cavity which was connected directly through the lead tube

to the air system. Pressure changes within the system produced movements in the diaphragm which caused alterations to the capacity of the transducer, thus imbalancing the bridge circuit.

A rectifier was provided to reduce the imbalanced A.C. output from the bridge to D.C. The amplitude of the D.C. potential so obtained (within the working range of the instrument) was a measure of the displacement of the diaphragm. The D.C. output from the electromanometer was fed into the final stage of the recording unit. In these experiments one of the D.C. amplifiers and a direct writing oscillograph of a Polyviso four-channel electrocardiogram were used.

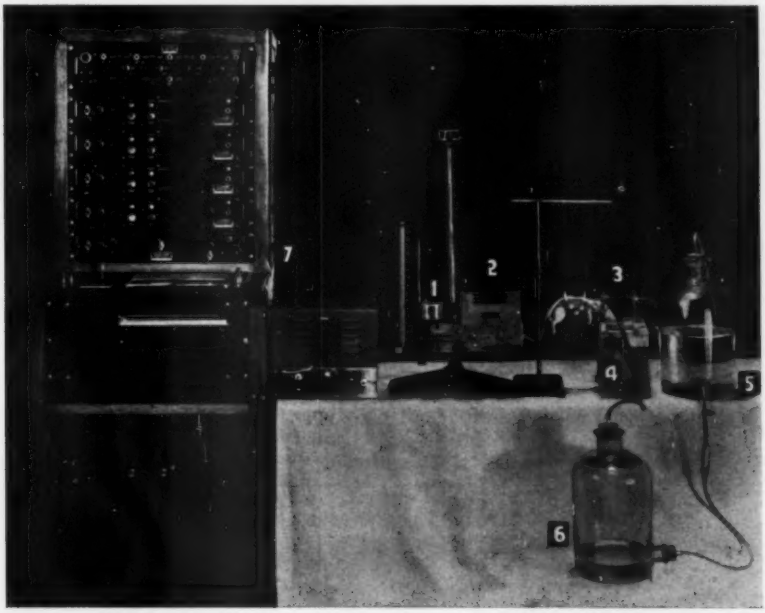

Fig. 2. Arrangement of the apparatus. 1. Transducer; 2. Electromanometer; 3. Respiration chamber; 4. Calibration syringe; 5. Water reservoir; 6. Air reservoir; 7. Recording unit.

The transducer was machined from brass, the insulating boss being made of polystyrene. The mould for pressing the diaphragms was constructed of brass. The metal foil used was 1/1,000th of inch in thickness. Fig. 4 shows the constructional details of the transducer.

In making a measurement, the system was first checked for any leaks, and recordings then made over short intervals varying from ½ to 1 minute. Calibration was effected by sealing the chamber and removing from it known volumes of air by means of a calibrated tuberculin syringe. The degree of needle deflection was thus determined for any change in air volume of the system. The removal of known volumes of water from the water reservoir was also used to check deflection against volume change.

For recording intrapleural pressures it was necessary to reduce the sensitivity of the recording system. This was done by using a thicker diaphragm, such that pressure changes of the order of 1/100th of an atmosphere would produce full-scale deflection. Direct connection with the pleural space recorded pressure changes graphically. Calibration was effected by inserting a water manometer in the circuit and calibrating pressure changes against deflection.

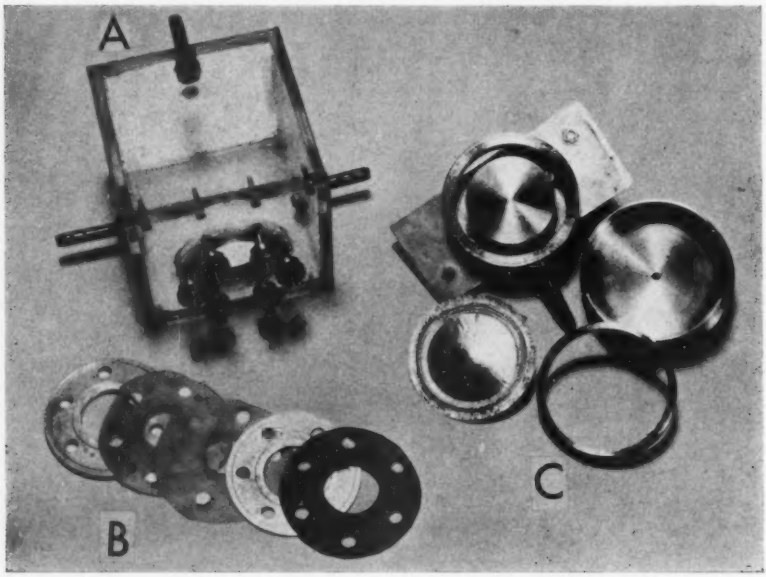

Fig. 3. (A) Respirometer chamber showing threaded studs to support aluminium collars.

(B) Aluminium supporting collars, with dental dam sealing collars.

(C) Exploded view of transducer. Base of transducer is shown attached to the plate which is fitted to the electromanometer. Cap, diaphragm and sealing ring are shown separately.

A fine gauge hypodermic needle flattened at the end with two countersunk holes drilled laterally was inserted through the thoracic wall between the pleural leaves. A small bubble of air introduced in the countersunk holes acted as a tonometer to record the pressure changes.

Intrapleural pressures in some animals were recorded graphically on a kymograph drum using a Marey's Tambour and manometrically on a water manometer. These records showed qualitative changes adequately, although system inertia and the lag and overswing in the water column of the manometer caused errors in absolute measurements.

All intrapleural pressures were recorded with the rat in the supine position and the needle located around the fifth to seventh intercostal space. Intrapleural pressures were recorded in normal, unanaesthetized, anaesthetized and phrenicectomized animals and in these rats with fluid injected into the opposite pleural cavity.

RESULTS.

The respiratory pattern, intrapleural pressure and pulmonary ventilation have been determined in groups of normal rats. The effects of nembutal anaesthesia, phrenicectomy, and intrapleural injection of fluid on these measurements have also been determined.

The respiratory pattern.

Normal rats. A characteristic diphasic type of respiratory pattern was produced by the normal unanaesthetized rat (Fig. 5 A and B). At slow recording speeds the wave form was smoothly sinusoidal. The tidal air in individual rats remained reasonably constant throughout the short periods of recording.

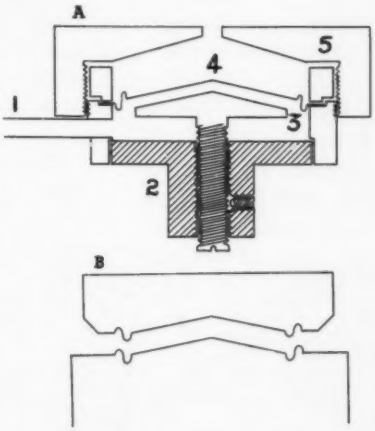

Fig. 4. (A) Cross section of the transducer. 1. Inlet tube; 2. Polystyrene insulating boss; 3. Adjustable stator plate; 4. Metal foil diaphragm; 5. Brass cap.

(B) Section of diaphragm mould.

At higher recording speeds the patterns showed smaller wave components of high frequency, whilst the overall form of the curve remained smoothly biphasic. These high frequency components varied in number in different patterns but were remarkably constant and characteristic throughout the pattern of individual rats. The frequency of these wave forms varied between 1/6th and 1/50th of a second. Some of these higher frequency components were due to heart beats, others were probably due to vibrations caused by air movement during the respiratory cycle.

In rapidly respiring unanaesthetized rats, half a respiratory cycle occupied time intervals varying from 0·16 to 0·5 of a second, i.e., respiratory rates of 60 to 190 per minute. Observations in normal rats in cages have indicated respiration rates higher than these recorded, as occurring commonly in active rats. The residual air remained constant during the recording of most rats. Variations in tidal air occurred usually as changes in the complementary air.

Effect of nembutal anaesthesia. Similar respiratory patterns to those of normal rats were recorded at moderate depths of nembutal anaesthesia. Respiration rates were slow due largely to a prolongation of the expiratory pause. At higher recording speeds accessory cycles appeared but these tended to be of smaller amplitude than in the unanaesthetized animals and the general wave form was smoother. As anaesthesia approached fourth stage, the respiratory amplitudes became less and the wave form flatter. Prolonged apnoeic periods occurred interspersed with respiratory cycles at varying and irregular time intervals.

During these prolonged periods of apnoea a pneumocardiac component could be readily recorded due to the heart beat.

Effect of phrenicectomy. In rats whose diaphragms had been paralysed by phrenicectomy two hours before, the patterns became very irregular (Fig. 6 A and B). High frequency recurring cycles accompanied a short gasping inspiratory effort. Tidal air and respiratory rates were reduced. The higher frequency components varied from 1/15th to 1/50th of a second and were of larger amplitude than those occurring in normal rats.

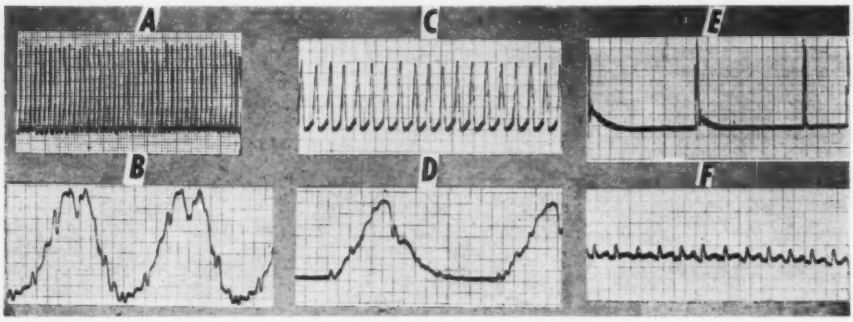

Fig. 5. Respiratory patterns. Upper series of tracings recorded at 5 mm./sec., lower series at 50 mm./sec. 1 division = 1 mm. Inspiration, up; expiration, down.

(A) (B) Normal rats.

(C) (D) Nembutal anaesthesia.

(E) (F) Deep nembutal anaesthesia. Recording (F) shows pneumocardiac component in an apnoeic period.

In rats two weeks after phrenicectomy the respiratory patterns had superimposed irregularities on diphasic curves, but the wave form resembled the normal pattern (Fig. 6E). The changes in respiratory stresses that accompany diaphragmatic paralysis are readily seen in Fig. 7. The normal rat breathing with hardly any intercostal or abdominal element shows little movement of the abdomen with respiration. There is a short period in the inspiratory phase when the diaphragm descends during which the chest wall is pulled inwards near the diaphragmatic attachments to the costal arch. The respiration of the phrenicectomized rat is strikingly different. The inspiratory phase is accomplished with an elevation and expansion of the whole costal arch and the abdominal wall is drawn inwards as the chest wall expands.

Intrapleural injection of fluid. In normal rats no appreciable changes occurred in the respiratory patterns when 6 ml./kg. Ringer-Locke solution were injected intrapleurally.

In animals after phrenicectomy, the injection of fluid, 6 ml./kg., caused

striking changes to the respiratory patterns (Fig. 6 C and D). In most of these animals within one hour of injection respirations became noticeably laboured and spasmodic. The pattern was characterized by numerous high frequency cycles which occurred synchronously with a terminal gasping inspiratory effort.

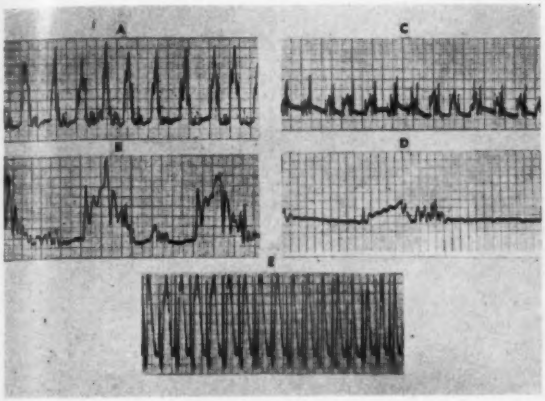

Fig. 6. Changes in respiratory patterns. Inspiration, up; expiration, down.

(A) (B) Phrenicectomized rat, 2 hours post-operative.

(C) (D) Phrenicectomized rat, 2 hours post-operative plus intrapleural injection of fluid, 6 ml./kg.

(E) Chronic phrenicectomized rat, 3 weeks post-operative.

Fig. 7. Photograph showing the respiration of the phrenicectomized rat, above, and the normal rat, below. The abdomen is drawn in passively as the costal arch is elevated and expanded during inspiration in the rat with paralysed diaphragm.

Later the pattern showed a progressive reduction in the amount of air moved with each inspiratory effort. The tidal air became small and the respiratory curve very flat. This change in respiratory pattern coincided with loss of respiratory reserve and progressive atalectasis of the pulmonary parenchyma.

Animals with diaphragmatic paralysis of longer standing showed no marked changes in the respiratory patterns following the injection of fluid, 6 ml./kg.

Intrapleural pressures.

Normal rats. Electromanometer recordings of static intrapleural pressures in rats under ether anaesthesia varied from —70 mm. of water at the end of inspiration to —10 mm. of water at the end of expiration (Fig. 8). These measurements are made in relation to atmospheric pressure which is considered as zero. Pleural pressure amplitudes were of the order of 50 mm. of water.

In normal rats allowed to recover from anaesthesia, static pressures varied from —40 mm. of water to —5 mm. of water. Amplitudes were much smaller, of the order of 30 mm. of water.

The effect of diaphragmatic paralysis. Manometric measurements and kymographic records of intrapleural pressures showed a significant increase in pleural pressures immediately following phrenicectomy. Table 1 shows the

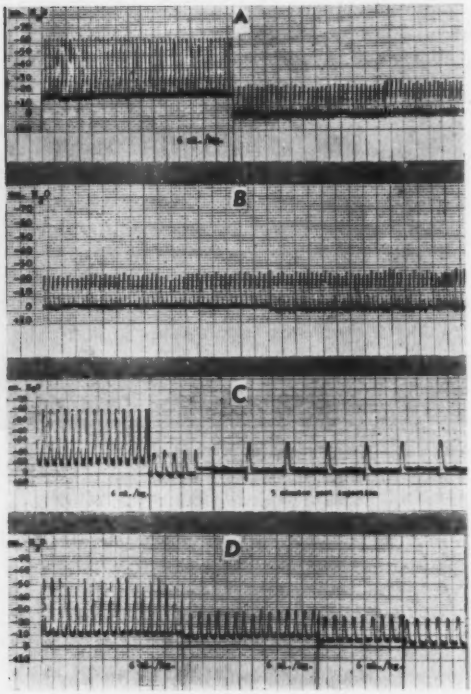

Fig. 8. Intrapleural pressure recordings. Inspiration, up; expiration, down.

(A) Effect of intrapleural injection of fluid in normal anaesthetized rat.

(B) Pressure recording in a normal unanaesthetized rat following the injection of 12 ml. of fluid intrapleurally.

(C) Effect of intrapleural injection of fluid in the rat 2 hours after phrenicectomy.

(D) Effect of injection of fluid in the rat 10 days after phrenicectomy.

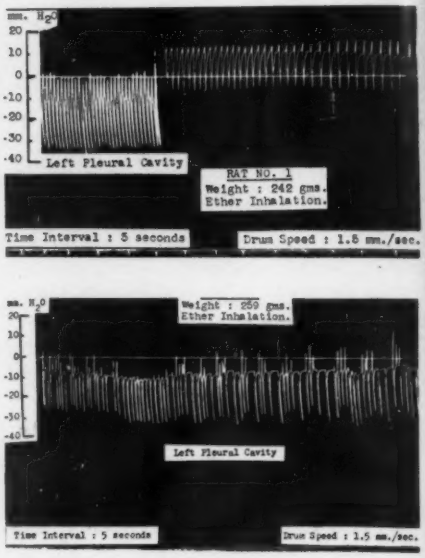

Fig. 9. The effect of phrenicectomy on the intrapleural pressure. Inspiration, down; expiration, up.

The upper tracing shows the pressure changes immediately following avulsion of the phrenic nerves.

The lower tracing shows the uneven type of intrapleural pressure amplitudes recorded in a rat 1 week after phrenicectomy.

effects of bilateral phrenicectomy on the intrapleural pressure. In normal rats values for the mean intrapleural pressure were —23·6 mm. of water with a mean amplitude of 32·7 mm. of water. Immediately following phrenicectomy the mean value rose to — 5·5 mm. of water with an amplitude of 25·8 mm. of water (Fig. 9).

TABLE 1.

The effect of phrenicectomy on intrapleural pressures. Results shown for 10 rats, mean weight 237 gm.

	Before phrenicectomy	After phrenicectomy
Respiration rate/minute		
Range	66 to 98	40 to 78
Mean	79·5	57
S.E. mean	± 3·52	± 4·44
Static inspiratory pleural pressure, mm. H_2O		
Range	— 30 to — 50	— 7 to — 30
Mean	— 40	— 18·9
S.E. mean	± 2·15	± 2·45
Static expiratory pleural pressure, mm. H_2O		
Range	0 to — 15	+ 15 to — 4
Mean	— 7·3	+ 7·9
S.E. mean	± 1·85	± 1·59
Amplitude, mm. H_2O		
Range	26 to 45	16 to 37
Mean	32·7	25·8
S.E. mean	± 2·12	± 2·44
Mean pleural pressure, mm. H_2O		
Range	— 18 to — 30	+ 4 to — 15
Mean	— 23·6	— 5·5
S.E. mean	± 1·7	± 1·46

Animals able to compensate successfully for the loss of diaphragmatic function showed intrapleural pressure values which were close to those of normal rats. Values had returned to normal levels one to three weeks after phrenicectomy. The intrapleural pressure pattern was more uneven in phrenicectomized rats than in normal rats. Animals failing to compensate successfully for the loss of diaphragmatic function died during the early period after phrenicectomy. Death was due to a failure on the part of accessory respiratory muscles to maintain an adequate pulmonary ventilation.

The effect of intrapleural injection of fluid. In anaesthetized normal rats the intrapleural injection of fluid, 6 ml./kg., produced an immediate reduction in the amplitude of the intrapleural pressure and an increase in its mean value. This was due chiefly to an increase in the mean static inspiratory pressure. Injection of fluid, 12 ml./kg., caused similar effects, the respiratory reserve of the normal rat being adequate to compensate for this volume of fluid.

In rats with recently paralysed diaphragms the injection of fluid, 6 ml./kg., caused considerable increases in the intrapleural pressures. The respiratory reserve in most animals was so severely reduced that fluid injections of this size produced progressive anoxia followed by death in one to two hours. The

amplitude of the intrapleural pressures was reduced immediately following injection as in normal rats. The mean intrapleural pressure approached atmospheric levels in these animals. Both inspiratory and expiratory static pressures were increased and in some rats the mean intrapleural pressure following injection was above atmospheric.

In rats phrenicectomized two weeks previously, intrapleural injection of fluid, 6 ml./kg., caused a similar increase in intrapleural pressure to that seen in normal rats. That compensation had occurred was evident from the fact that these rats could maintain a subatmospheric intrapleural pressure and ventilate successfully when fluid was injected at the rate of 12 ml./kg.

TABLE 2.

Changes in respiration rate, tidal air and minute volume following phrenicectomy, nembutal anaesthesia and intrapleural injection of fluid.

	Weight gm.	Respiration rate/minute	Tidal air ml.	Minute volume ml./minute
Normal				
Range	101–150	116–182	0·5–0·9	75·0–110·4
Mean (6)	131·5	152·5	0·64	95·5
S.E. mean	± 8·3	± 11·18	± 0·057	± 6·03
2 hours post phrenicectomy				
Range	98–134	120–140	0·43–0·57	17·6–68·4
Mean (6)	115·8	63·3	0·48	31·3
S.E. mean	± 5·55	± 11·96	± 0·024	± 9·45
10 days post phrenicectomy				
Range	114–150	70–130	0·49–1·35	53·5–94·5
Mean (6)	132·7	94·8	0·77	66·5
S.E. mean	± 5·25	± 8·95	± 0·129	± 6·42
Nembutal anaesthesia				
Range	142–155	21–64	0·44–1·36	20·0–46·1
Mean (6)	151·0	42·5	0·82	31·0
S.E. mean	± 2·08	± 6·04	± 0·171	± 4·74
Normal, intrapleural injection 6 ml./kg.				
Range	120–144	90–120	0·60–1·02	72·0–91·8
Mean (3)	132·7	108	0·71	79·7
S.E. mean	+ 6·96	± 9·16	± 0·321	± 6·14
2 hours post phrenicectomy, intrapleural injection 6 ml./kg.				
Range	136–160	20–62	0·28–0·6	6·2–37·2
Mean (6)	150	33·7	0·4	15·2
S.E. mean	± 3·78	± 6·35	± 0·054	± 4·93
10 days post phrenicectomy, intrapleural injection 6 ml./kg.				
Range	142–160	68–130	0·54–0·80	54·5–70·2
Mean (2)	151	99	0·67	62·3
S.E. mean	± 9·0	± 31·0	± 0·42	± 7·93

Pulmonary ventilation.

Changes occurring in the respiration rate, tidal air and pulmonary ventilation after phrenicectomy, nembutal anaesthesia and intrapleural injection of fluid are shown in Table 2. The changes occurring immediately following diaphragmatic paralysis are evident. Both respiratory rate and tidal air were reduced, which in turn caused a reduction in the minute pulmonary ventilation. In rats examined 10 days after diaphragmatic paralysis these respiratory functions had increased significantly towards normal values. An even greater reduction in these functions occurred in recently phrenicectomized rats following the intrapleural injection of fluid. The extent of the reduction made adequate ventilation extremely difficult in these animals.

The ability of both normal and chronically paralysed rats to compensate successfully when fluid was introduced into their pleural cavities was seen from the small reduction in minute ventilation that followed the injection of fluid. Nembutal anaesthesia caused a reduction in minute ventilation that became increasingly greater as the stage of bulbar paralysis was approached.

DISCUSSION.

Our results have shown higher respiratory rates in normal rats than those recorded by Guyton (1947) and McCutcheon (1951) using various spirometric and respirographic techniques. This could be due to increased excitement in our animals during recording but, from observation of normal rats in cages, respiratory rates higher than those recorded respirographically are readily counted. The respiration of the unanaesthetized rat is typically rapid and shallow when the animal is active.

The striking reduction in respiratory rate, tidal air and minute ventilation with phrenicectomy emphasizes the fundamentally diaphragmatic nature of the rat's respiratory mechanism. In such a pronograde animal the intercostal element would seem to be poorly developed and diaphragmatic paralysis encroaches to a large extent on the animal's respiratory reserve. The reduction in pulmonary ventilation is noticeable in the prolonged period of recovery from anaesthesia following phrenicectomy.

In man, various workers have studied the effects of unilateral paralysis on respiratory volumes. Vaccarezza *et al.* (1948) examined the respiratory changes following phrenicectomy in tuberculous patients and reported reductions to a variable degree in the oxygen consumption, reserve air, complementary air, vital capacity and pulmonary ventilation. Brea and Ferrari (1935) reported similar findings in tuberculous patients and noted that, with the development of compensatory mechanisms, these changes reverted to normal values one month after surgical intervention. Werner (1930) reported a reduction of 33 p.c. in the vital capacity in humans immediately following unilateral phrenicectomy, with

a return to normal pre-operative levels in from two to eight weeks. Gianotty and Ceruti (1930) recorded reductions in pulmonary volumina after unilateral and bilateral phrenicectomy in tracheotomized dogs. The effect of introducing fluid into the pleural cavity of animals recently deprived of diaphragmatic function accentuates the reduction already made in the respiratory reserve. Changes occurring in the pulmonary volumina are reflected in changes within the pleural space itself. The pleural pressure changes following phrenicectomy in rabbits were investigated by Custo (1938). Custo found the intrapleural pressure to increase following phrenicectomy and to return to normal values a week or so later. He concluded that the restoration of the intrapleural pressure to normal levels was due to an increase in the tone of other respiratory muscles. The pronounced increase in pleural pressure in the rat following paralysis of the diaphragm represents a reduction in the potential tension on the visceral and parietal pleural surfaces. This would feature a low pulmonary stress associated with atalectasis of the lung tissue. Osugi (1938) reported the collapse to be confined chiefly to the inferior lobes of the lungs. Atalectasis becomes progressively more extensive with the addition of fluid to the pleural space. The subatmospheric phase of the pleural pressure becomes less in degree and duration with a consequent failure of the lungs to inflate and provide adequate oxygenation.

Changes in venous pressure following phrenicectomy were discussed by Lusena (1935). The increased value of the intrapleural pressure was reflected in an elevated venous pressure. Christie and Meakins (1934) concluded that an increase in the intrapleural pressures was an important factor in elevating the venous pressures.

Blood flow to the heart is influenced largely by the aspiration effect of the intrapleural pressure. Similarly the flow of lymph within the large valved lymphatic trunks of the thorax would be affected by changes in intrapleural pressures and by changes in the venous pressure at the lymphatic venous confluence.

Changes occurring in the respiratory patterns are more difficult to interpret. The respiratory pattern of the normal rat is a diphasic curve, comparatively smooth in form. This is considered to represent to a large extent the functional activity of the diaphragm. This pattern becomes even smoother under anaesthesia. In animals with diaphragmatic paralysis respiratory function is of necessity transferred to the intercostal muscles and the patterns become very irregular. Osugi (1938) stated that phrenicectomy was followed by increased movement of the thoracic walls. Rats with recently paralysed diaphragms have difficulty in maintaining their inspiratory static pleural pressures and their respirations are laboured and badly co-ordinated.

SUMMARY.

A modified respirometer for measuring the respiration rate, tidal air, pulmonary ventilation and intrapleural pressure in the rat is described.

The changes occurring in the respiratory pattern and pulmonary volumina following phrenicectomy, anaesthesia and intrapleural injection of fluid have been measured.

Following phrenicectomy there was an increase in the mean intrapleural pressure from about —24 mm. of water to —5 mm. of water. This increase was transitory and returned to normal values one to three weeks later. Similar increases in intrapleural pressures were recorded following the intrapleural injection of fluid at the rate of 6 ml./kg.

The tidal air and pulmonary ventilation were significantly reduced following diaphragmatic paralysis. The minute volume fell from about 95 ml./minute in the normal rat to 30 ml./minute following diaphragmatic paralysis. The injection of fluid in the acutely phrenicectomized rat reduced the minute ventilation still further to about 15 ml./minute.

In rats with diaphragmatic paralysis of a few weeks' duration respiratory function returned towards normal levels. Intrapleural pressure values were similar to those of normal rats and the pulmonary ventilation and respiratory reserve had increased to approximately that of normal rats.

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THE REMOVAL OF FREE RED BLOOD CELLS FROM THE PERITONEAL CAVITY OF ANIMALS[1]

by F. C. COURTICE, J. HARDING AND A. W. STEINBECK

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Particles of various size have been extensively used in investigations of lymphatic absorption from the peritoneal cavity. Of these particles red blood cells have often been employed because of the practical importance of the absorption of extravasated blood at operation or after trauma and of the possible value of intraperitoneal blood transfusions. Attention has been focussed largely on the manner in which the particles enter the diaphragmatic lymphatics and little information is available concerning the lymph as it enters the blood-stream. Florey and Witts (1928) collected lymph from the thoracic duct after the peritoneal injection of whole blood, and were not impressed by the power of the lymphatics to remove red cells from the peritoneal cavity. It has since been shown, however, that the diaphragmatic lymphatics drain mainly into the right lymph duct (Courtice and Steinbeck, 1950), so it was thought worth while to collect lymph from this duct as well as from the thoracic duct. In addition it was decided to investigate the rate of absorption of blood and the relative importance of phagocytosis and lymphatic absorption in clearing the peritoneal cavity of red cells.

METHODS.

Rats, guinea-pigs, rabbits and cats were used. To determine the rate of removal of red cells, heparinized whole blood or a suspension of washed red cells in Ringer-Locke solution was introduced into the peritoneal cavity of rats, guinea-pigs and rabbits. The method was that used previously (Courtice and Steinbeck, 1951) and the volume introduced was 20 ml. per kg. body weight. These animals were anaesthetized only for this procedure, rats and guinea-pigs with ether and rabbits with intravenous nembutal. They rapidly recovered from the anaesthetic and moved normally in their cage. At intervals after this introduction of red cells, the animals were killed, rats and guinea-pigs with coal gas and rabbits with intravenous nembutal, and the blood remaining in the peritoneal cavity recovered. By means of haematocrit determinations of the blood introduced and that recovered, the volumes of cells and of plasma absorbed were measured.

White cell counts were made on the peritoneal fluid during the first 5 to 8 hours before clumping of the large macrophages made this impossible. Differential counts and the phagocytosis of red cells were studied in films stained by the Jenner-Giemsa method.

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

Histological examination of the apical mediastinal lymph nodes was made mainly in the rat. The whole of the apical portion of the thorax was fixed in Bouin's fluid. After fixation the small lymph nodes were cut away together with some adjacent thymus tissue. In this way about three to five lymph nodes were embedded in one block for section. Sections were stained with haematoxylin and eosin or by Turnbull's Blue method to detect the presence of iron.

Lymph collection was made in cats, anaesthetized with nembutal, from the thoracic duct and the right lymph duct before and after the introduction of blood into the peritoneal cavity. These ducts were cannulated in the lower part of the neck just before the ducts entered the veins.

To determine the relative importance of phagocytosis in the removal of blood from the peritoneal cavity, the same volume of blood was introduced intravenously or intraperitoneally in groups of rats, and the change in circulating haemoglobin determined on tail-vein samples. The rats were unanaesthetized except for the introduction of the blood and taking the blood samples when light ether anaesthesia was used.

RESULTS.

Rate of removal of homologous blood.

The rates at which homologous red cells were removed from the peritoneal cavity of the rat, rabbit and guinea-pig are shown in the lower part of Fig. 1. These rates varied considerably in the three animals, being fastest in the rat and slowest in the guinea-pig. The relative speed of absorption of plasma was also fastest in the rat and slowest in the guinea-pig, as can be seen from the upper part of Fig. 1.

Rate of removal of heterologous blood.

Some investigators have used foreign red cells to study the lymphatic absorption of particles from the peritoneal cavity. In view of the work of Courtice and Steinbeck (1951) on foreign protein absorption from the peritoneal cavity, it was decided to determine the removal of human and rabbit whole blood and also washed red cells suspended in Ringer-Locke solution from the peritoneal cavity of the rat. The results are shown in Figs. 2 and 3. In Fig. 2 the foreign plasma effect on the capillary permeability is evident. The volume of plasma in the peritoneal cavity greatly increased during the first few hours. When the foreign protein reaction ceased the absorption of the plasma fraction was rapid (*cf.* Lake, Simmonds and Steinbeck, 1953). In these circumstances the human and rabbit red cells were much more slowly absorbed than were the homologous rat cells.

In the experiments represented in Fig. 3 red cells were washed twice with, and then resuspended in, Ringer-Locke solution to give a haematocrit value the same as in the original blood. In these experiments in the absence of the foreign plasma reaction it was found that human, rabbit and rat cells were all absorbed at the same rate. The foreign cells themselves, therefore, appeared to act merely as inert particles as far as their absorption into the lymphatics was concerned, and had no effect on the capillary permeability as had foreign plasma.

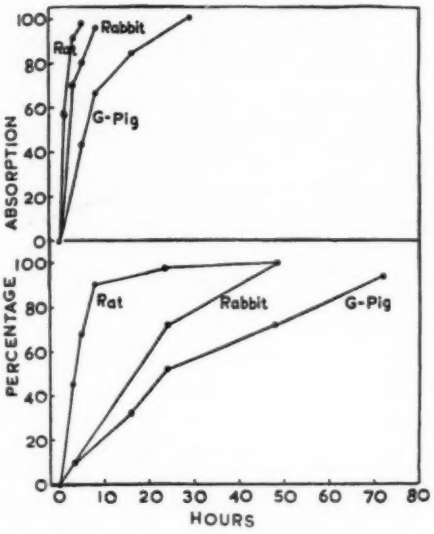

Fig. 1.

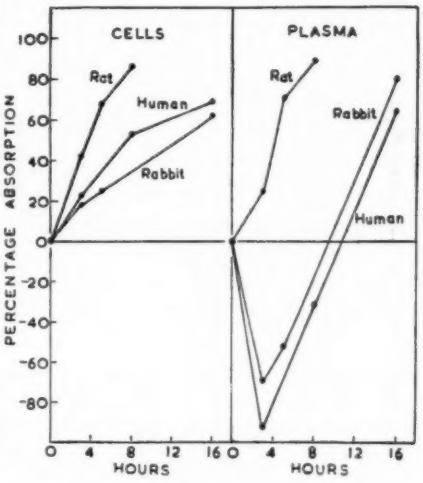

Fig. 2.

Fig. 1. Lower graph. The rate of removal of red cells from the peritoneal cavity of the rat, rabbit and guinea-pig after the intraperitoneal injection of 20 ml./kg. heparinized, homologous blood.

Upper graph. The rate of removal of fluid from the peritoneal cavity of the rat, rabbit and guinea-pig after the intraperitoneal injection of 20 ml./kg. heparinized, homologous plasma. Each point represents the mean of 3 to 6 experiments.

Fig. 2. The rate of removal of red cells and of plasma from the peritoneal cavity of the rat after the intraperitoneal injection of 20 ml./kg. of rat, human and rabbit blood. A negative absorption means that fluid has been added to that introduced. Each point represents 3 to 6 experiments.

After the introduction of whole blood into the peritoneal cavity there was at first a dilution. This is shown by the changes in haematocrit value of the blood removed from the peritoneal cavity at various intervals (Table 1). In the case of homologous blood the early dilution was due to the osmotic effect of the proteins in the plasma. A non-protein fluid, therefore, passed into the peritoneal cavity to lower the protein level. With heterologous blood, the dilution was very much greater mainly due to the increased capillary permeability from which a protein-rich fluid passed into the peritoneal cavity (Courtice and Steinbeck, 1951). After this initial phase, however, the plasma was more rapidly absorbed than the cells, so that the haematocrit of the peritoneal blood rose until ultimately the whole of the parietal and visceral peritoneum was covered by a layer of sticky red cells. Absorption of these red cells then appeared to slow down, but within a further 24 hours they had all been removed

leaving no trace of the earlier presence of blood. It is remarkable how such a layer of red cells could be moved up to the diaphragm and absorbed in such a relatively short time.

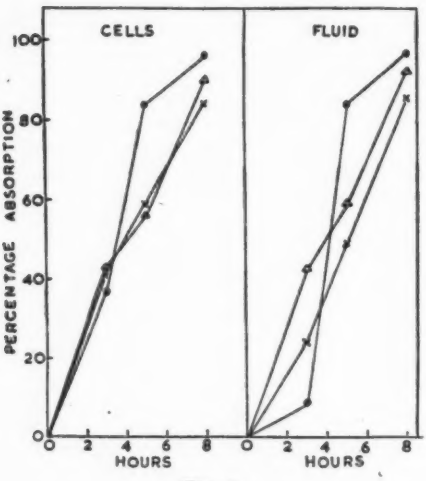

Fig. 3.

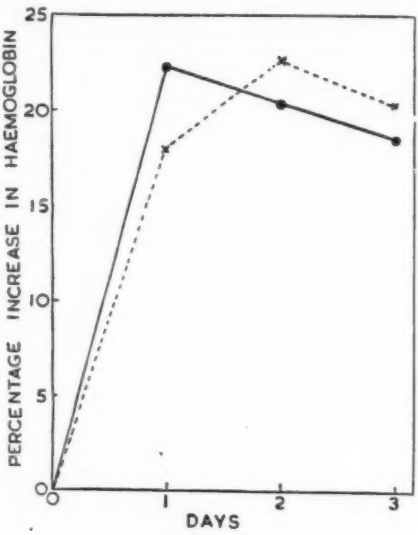

Fig. 4.

Fig. 3. The rate of removal of red cells and of fluid from the peritoneal cavity of the rat after the intraperitoneal injection of 20 ml./kg. of washed rat, human and rabbit red cells suspended in Ringer-Locke solution. Each point represents 2 to 6 experiments.

● Rat. × Rabbit. △ Human.

Fig. 4. The increase in the haemoglobin concentration of the circulating blood of the rat after the intravenous or intraperitoneal injection of 20 ml./kg. of homologous blood. Average figures for 10 animals in each group.

● Intravenous. × Intraperitoneal.

TABLE 1.

The haematocrit values of the blood in the peritoneal cavity on injection and at intervals after the introduction of 20 ml./kg. of heparinized whole blood in the guinea-pig and rat. Mean figures for groups of 3 to 6 animals at each time.

	Guinea-pig			Rat					
	Homologous blood			Homologous blood			Rabbit blood		
Time hours	Injected	Remaining Actual	p.c.	Injected	Remaining Actual	p.c.	Injected	Remaining Actual	p.c.
3	37·3	34·0	91·1	42·7	37·4	87·7	33·6	20·2	60·0
5	38·9	34·7	89·4	42·7	46·9	109·5	33·6	19·8	59·0
8	—	—	—	42·1	49·2	117·0	—	—	—
16	36·5	42·4	116·3	—	—	—	33·6	48·0	142·8
24	40·5	63·7	157·0	—	—	—	—	—	—

During the passage of the red cells along the parasternal lymphatics, there was sometimes an extravasation of these cells from the lymphatics to give rise to haemorrhages beneath the pleura. The red cells appeared first to dam back along a tributary of the parasternal lymph trunk and then to escape through the lymph capillaries of that region beneath the pleura. In some instances small areas of extravasated red cells were seen confined to individual intercostal spaces; in others the whole area beneath the sternum was dark red due to extravasated red cells from the parasternal lymph trunks and their tributaries. Free red cells were also present in the pleural cavity. This phenomenon was seen both with homologous and heterologous blood, but more frequently with heterologous. Microscopic section showed that it was whole red cells and not haemoglobin from lysed cells that escaped from the lymphatics. A similar picture was observed during the absorption of plasma (Courtice and Steinbeck, 1950, 1951).

With homologous blood no haemolysis of the red cells occurred either in the peritoneal cavity or in the circulating blood after absorption. With heterologous blood, however, some haemolysis occurred in the peritoneal cavity and when the cells entered the circulation they underwent haemolysis which seemed to vary with different specimens of blood. The rat's plasma agglutinates all foreign red cells, and in some cases the resulting haemolysis was intense with the plasma and the urine dark red due to the presence of haemoglobin. Once the haemoglobin from the lysed foreign cells was excreted, however, the rats appeared quite normal with no macroscopic signs of the peritoneal injection of heterologous blood.

The local peritoneal reaction.

After the introduction of blood into the peritoneal cavity there is a local cellular reaction in which there is mainly an accumulation of neutrophils and of large mononuclear macrophages. In the rat mast cells and eosinophils also appear (*cf.* Buxton and Torrey, 1906; Webb, 1931). In the present series of experiments the total white cell count was determined up to 5 to 8 hours after the introduction of the blood, but after this time the large macrophage cells clumped together so that it was impossible to count them accurately. White cell counts were made in several experiments in rats and in guinea-pigs, the average results of which are given in Table 2.

Although there was a considerable outpouring of large mononuclear macrophages, it appeared that they did not play a large part in the removal of the red cells. In the counts made, the number of macrophages containing red cells varied considerably, but on the average only 6 p.c. of these cells contained one or more red cells. On this basis and from a consideration of the actual number of macrophages present, it was evident that this method of removal of a fairly large amount of blood from the peritoneal cavity would be slow.

TABLE 2.

White cells in peritoneal fluid at intervals after introduction of 20 ml./kg. whole blood into the peritoneal cavity of the rat and guinea-pig. Mean figures for 3 animals in each group.

		Differential count as percentage of total				
	Total c/mm.	Neutrophils	Lymphocytes	Mast	Large mononuclears	Eosinophils
RATS						
Rat blood						
Injected	6,400	14·0	84·0	—	—	2·0
Recovered 3 hours	14,000	36·0	8·0	5·1	43·7	7·0
5 hours	22,700	37·3	6·0	3·0	48·3	5·5
Human blood						
Injected	6,800	—	—	—	—	—
Recovered 3 hours	7,150	41·5	25·5	3·0	27·5	3·0
5 hours	8,900	36·5	13·2	2·2	40·1	5·0
8 hours	7,650	49·5	8·3	1·1	38·3	2·6
Rabbit blood						
Injected	4,300	78·0	21·5	—	—	—
Recovered 3 hours	9,900	71·2	12·2	0·7	15·5	0·4
5 hours	9,600	77·8	13·0	0·2	7·5	0·5
GUINEA-PIGS						
Guinea-pig blood						
Injected	2,200	—	—	—	—	—
Recovered 3 hours	4,300	45·0	51·0	—	2·1	—
5 hours	4,750	61·0	14·0	—	20·0	—
16 hours	—	49·0	11·0	—	35·0	—
48 hours	—	24·0	20·0	—	51·0	—

Lymphatic absorption of blood.

It has been shown by Courtice and Steinbeck (1950) that the right lymph duct carries most of the lymph from the diaphragmatic lymphatics and that in the cat as in the dog this duct may vary considerably and so may be difficult to cannulate. In the present series of experiments, cannulation of the right lymph duct was sometimes found impossible because the efferent vessels from the mediastinal apical lymph nodes were found, *post mortem*, to enter the vein very low down in the thorax or form a plexus of fine vessels which might pass either to right or left side. Even though these vessels passed occasionally mainly to the left side they entered the vein separately from the thoracic duct. For these technical reasons it was only when the efferent vessels from these nodes formed a single right lymph duct that lymph could be collected.

The right lymph duct and thoracic duct were both cannulated in three cats and the thoracic duct alone in others. The detailed results of one of the experiments in which both ducts were cannulated are given in Table 3. In this experiment clear lymph came from the right lymph duct and opalescent lymph from the thoracic duct, in both cases containing very few red cells.

TABLE 3.

The red and white cell counts in the lymph from the right lymph duct and thoracic duct of a cat before and after the intraperitoneal injection of 10 ml./kg. homologous blood.

		Right lymph duct							Thoracic duct		
					White cells in lymph						
Time	Air breathed	Lymph flow ml./hr.	Red cells in lymph/cmm.	Total /cmm.	N p.c.	L p.c.	M p.c.	E p.c.	Lymph flow ml./hr.	Red cells in lymph /cmm.	White cells in lymph Total/cmm.
Before	Room air	0·7	80	2,310	—	—	—	—	7·5	450	4,120
After											
0-½ hr.	Room air	0·8	90	2,370	—	—	—	—	5·2	1,300	4,200
½-¾ hr.	„	0·8	503,000	4,900	37	61	—	2	—	—	—
¾-¾ hr.	„	0·8	2,650,000	7,530	23	50	4	13	7·2	1,625	4,330
¾-1 hr.	„	1·2	3,680,000	11,750	54	38	3	5	7·2	1,950	4,050
1-1½ hr.	„	1·0	4,170,000	10,400	41	38	11	8	8·0	2,180	4,280
1½-2 hr.	„	1·0	4,530,000	9,850	51	33	5	11	7·0	5,700	3,630
2-2½ hr.	5 p.c. CO_2	2·8	5,550,000	4,250	63	18	10	7	14·4	68,000	8,030
2½-2½ hr.	„	4·4	5,560,000	3,700	50	33	12	4	14·4	90,000	6,200
2½-3 hr.	Room air	2·0	5,550,000	4,050	67	19	12	—	3·4	108,000	7,050
3-3½ hr.	„	2·2	4,840,000	3,850	64	18	7	11	4·8	93,500	4,850

N = Neutrophils. L = Lymphocytes. M = Mononuclears. E = Eosinophils.

Blood introduced: 8,020,000 red blood cells/cmm.
10,000 white blood cells/cmm.

After the introduction of 10 ml./kg. of cat's blood into the peritoneal cavity, the red cell content of the lymph in the right lymph duct rose rapidly until the lymphatic resembled a vein from which blood emanated. Breathing room air the red cell content of the lymph rose to 4,530,000 per cmm., and on increasing the diaphragmatic movement with 5 p.c. CO_2, this content rose to 5,550,000 per cmm., and the flow also increased from 1 ml./hour to 4·4 ml./hour. The collection of this whole blood from a lymph vessel was truly remarkable, and in all three animals the picture was similar.

The thoracic duct lymph on the other hand never contained very many red cells. The red cell content of the thoracic duct lymph in other experiments rose to 11,000, 97,000 and 300,000 per cmm., but never approached the levels reached in lymph from the right lymph duct.

It is evident from these experiments that the lymph from the diaphragmatic lymph vessels ultimately passed in the main into the so-called right lymph duct and not into the thoracic duct. Collection of lymph from the thoracic duct can, therefore, give a very erroneous picture of the power of the lymphatics in removing blood from the peritoneal cavity.

The red cells in the lymph from the right lymph duct were always normal in appearance and there was no haemolysis. On standing or centrifuging the red cells settled leaving a perfectly clear lymph with no trace of haemolysis. The white cell count of the lymph varied as absorption continued. At first

before the introduction of the blood the cells were practically all lymphocytes, but as absorption progressed neutrophils predominated and mononuclears and eosinophils were also present. Whether these latter cells were absorbed from the peritoneal cavity or entered the lymph in the lymph nodes is not known.

Phagocytosis of red cells in the lymph nodes.

From the above experiments it would seem that the lymph nodes in the path from the peritoneal cavity are not very efficient filters. Histological examination of these lymph nodes in the rat, however, taken at intervals from 3 hours to 12 weeks after the intraperitoneal injection of whole blood showed that the lymph sinuses through which the lymph passed soon became filled with macrophages choked with red cells. These large macrophages, each containing many red cells, appeared to block the lymph sinuses, but the lymph flow had been shown to continue.

Although the lymph nodes contained so many macrophages engorged with red cells, the actual number of red cells held up compared with those passing through the nodes was extremely small. The pumping action of the diaphragm and other muscles of respiration forced the cells on through the nodes at a rapid rate. Even when very much smaller volumes of blood were introduced the red cells rapidly passed through the lymph nodes into the circulation (Morris, 1953).

Once the red cells were engorged by the macrophages they were rapidly broken down. The Turnbull Blue stain for iron was faintly positive in these macrophages as early as 5 to 8 hours. After the red cells were broken down, some of the iron remained in the macrophages for at least 12 weeks. If the animals were bled repeatedly, however, to keep the haemoglobin level about 70 p.c. of normal, the iron disappeared from the macrophages much more rapidly. Groups of two or three animals in the control and bled series were killed 3 days and 1, 2, 4, 6, 8, 10 and 12 weeks after the peritoneal introduction of blood. During the first week there was no detectable difference between the iron content of the lymph node macrophages in each series, 17 out of 19 glands containing iron in the control series and 14 out of 15 in the bled series. From two to twelve weeks there was a significant difference, 37 of 42 glands in the control series and only 9 of 46 glands in the bled series containing iron.

The rise in circulating haemoglobin after intravenous and intraperitoneal blood injection.

In two groups of rats, 10 in each group, 20 ml./kg. of rat blood was injected intravenously in the tail vein in one group and intraperitoneally in the second group. The haemoglobin concentration of the tail vein blood was determined before the injection and at 24, 48 and 72 hours after the injection. In a third group the haemoglobin was determined at the same time intervals but no blood was injected. The rats were all approximately the same weight and age and

the average haemoglobin content of the circulating blood was initially 14·9 gm. p.c. in the control group, 15·5 gm. p.c. in the intravenous group and 15·3 gm. p.c. in the intraperitoneal group. The loss of blood from the tail vein in sampling was kept to a minimum and as near as possible the same in each group. In the control group the haemoglobin content of the tail vein blood fell to 97·6 p.c. of its initial value on the first day, 95·9 p.c. on the second and 95·0 p.c. on the third. These corrections have been made to the results obtained after the intravenous and intraperitoneal injection of blood, given in Fig. 4. It is evident that the ultimate effect of these transfusions on the circulating haemoglobin was the same. These experiments confirmed that the actual volume of red cells phagocytosed, either locally in the peritoneal cavity or in the lymph nodes through which the absorbed cells passed, was very small.

DISCUSSION.

The method by which particles pass through the peritoneum of the diaphragm into the underlying lymph vessels has been controversial since von Recklinghausen (1862) first postulated the presence of stomata. Cunningham (1926) in summarizing the work on this problem up to that time said that "the earlier work all tended to establish the concept of the presence of actual preformed physical openings between the peritoneal cavity and the diaphragmatic lymphatics. This idea was gradually eliminated and in its place the concept of potential physical openings between the cells was offered. In turn this hypothesis is being replaced by one which assumes that most, if not all, of the particulate material that is absorbed from the peritoneal cavity passes directly through the living cytoplasm of the mesothelial cells." Since this time the controversy has continued. Allen (1936) has reaffirmed the presence of stomata by using frog, chick and mouse erythrocytes and India ink in the mouse. He found the stomata to be from 2 to 6μ in diameter and gave evidence to suggest that some may be from 10 to 15μ in diameter. Simer (1948) on the other hand concluded that in white rats small particles such as India ink forced their way between the contiguous borders of the mesothelial cells of the peritoneum and the endothelial cells of the lymph vessels, but he found that frog erythrocytes and other particles larger than 7μ in diameter were unable to enter the diaphragmatic lymph plexus.

In the present series of experiments it has been shown that rat, rabbit and human red cells, the average diameters of which in the fresh state vary from 7 to 8μ, were all absorbed into the lymphatics at about the same rate in the rat, when they were washed and suspended in Ringer-Locke solution. When 20 ml./kg. of whole rat blood or washed cells was injected into the peritoneal cavity, practically all had been absorbed in 24 hours. Particles of this size, therefore, must be able to pass fairly freely through the diaphragmatic peri-

toneum, although the rate is somewhat slower than that of the protein molecules which have an equatorial diameter of about $^1/_{2,000}$ that of a red cell. In the rabbit the absorption was somewhat slower than in the rat, and in the guinea-pig the rate was slower still. Lymphatic absorption of plasma from the peritoneal cavity was also much slower in the guinea-pig than in the rat. This may be due either to a different anatomical arrangement of the lymphatics of the diaphragm, or to differences in diaphragmatic movement, since it is diaphragmatic movement that is mainly responsible for lymphatic absorption from the peritoneal cavity.

The relative ease with which the red cells pass into the lymphatics is well shown in the cell content of the lymph from the right lymph duct. The red cells have to pass through the peritoneum and through the lymph nodes, but they enter the blood stream without any apparent damage or haemolysis. Although other authors have shown by various methods that absorbed red cells enter the circulation intact (Hahn, Miller, Robscheit-Robbins, Bale and Whipple, 1944; Hedenstedt, 1947; McKee and Stewart, 1950), the power of the contracting diaphragm to absorb these large particles appears the more remarkable when lymph containing from 5 to 6 million red cells per cmm. is observed flowing from the cannulated right lymph duct.

The part played by the free macrophages in the peritoneal cavity in removing the red cells seems from these experiments to be almost insignificant. Buxton and Torrey (1906) described in guinea-pigs the phagocytosis of chicken red cells. They found that the macrophages were stuffed full of chicken corpuscles. In the present experiments the number of macrophages containing obvious red cells was small, on an average 6 p.c., and from the numbers of macrophages present (Table 2), it is evident that removal of free red cells by this method was negligible compared with that by lymphatic absorption. The macrophages in the lymph nodes through which the blood passed, on the other hand, became grossly engorged with red cells. From a consideration, however, of the total size of these lymph nodes compared with the volume of blood passing through them, it again becomes apparent that removal by this method of phagocytosis was also insignificant. These facts were borne out by the rise in haemoglobin observed after intravenous and intraperitoneal injections of blood into the rat.

The leakage of red cells which sometimes occurred as they passed through the anterior mediastinum may be in part due to a partial bottle-neck in the lymph nodes and to the negative intrathoracic pressure. Varying degrees of leakage were observed. In some instances the lymph trunks were merely engorged with red cells; in others some of the intercostal lymphatic tributaries of the parasternal lymph trunks were engorged while in others the red cells escaped from the lymph capillaries along an intercostal lymphatic. Sometimes

only one intercostal space was affected while in other instances the whole substernal area contained extravasated blood beneath the pleura, and free blood cells were present in the pleural cavities. The picture with red cells was exactly comparable with that observed by Courtice and Steinbeck with plasma proteins.

SUMMARY.

The rate of removal of red blood cells from the peritoneal cavity was determined in the rat, rabbit and guinea-pig. It was fastest in the rat and slowest in the guinea-pig.

Lymph was collected from the right lymph duct and thoracic duct of the cat before and after the intraperitoneal injection of homologous blood. Most of the red cells passed into the right lymph duct, which contained up to 5,500,000 red cells per cmm. These red cells emerged from the right lymph duct without any change or lysis.

The removal of red cells from the peritoneal cavity by phagocytosis locally or in the mediastinal lymph nodes was insignificant compared with the lymphatic absorption.

During the passage of the red cells along the parasternal lymph trunks, some of the red cells occasionally leaked out of the lymph tributaries of these trunks to give an extravasation of red cells beneath the pleura or into the pleural cavities.

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THE EFFECT OF DIAPHRAGMATIC MOVEMENT ON THE ABSORPTION OF PROTEIN AND OF RED CELLS FROM THE PLEURAL CAVITY[1]

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The removal from the pleural cavity of plasma proteins, labelled with the dye T1824, was investigated in rabbits and cats by Courtice and Simmonds (1949). They showed that the protein was removed by the lymphatics and that the rate of removal in rabbits was fairly rapid, 6 ml./kg., of homologous plasma being absorbed within 24 hours. This rate was about the same as that for the removal of the same volume of 0·9 p.c. NaCl. It was also shown that anaesthesia slowed down the absorption and that increased respiratory movement, brought about by inhalation of 7 p.c. CO_2, greatly accelerated absorption.

Cooray (1949) using radiopaque dyestuffs of various viscosities studied the path of the material injected into the pleural cavity of the normal rat and in rats with the diaphragm paralysed. He concluded that diaphragmatic paralysis caused by phrenicectomy, pneumothorax or pneumoperitoneum definitely delayed absorption. His criteria of retarded absorption were based on the visual record of a slowing in movement of the contrast agents towards the retro-cardiac mediastinal folds, which he regarded as the pleural absorbing surfaces. Long standing diaphragmatic paralysis led to minimal movements of the material within the thorax and presumably to minimal absorption.

In order to investigate further the absorptive mechanisms of the pleural cavities, it was decided to study the removal of plasma proteins labelled with the dye, T1824, and of red cells labelled with P^{32} from the pleural cavities of rats, and to ascertain the effect of respiratory movements on the rate of absorption. Plasma proteins and red cells have been used because they are the naturally occurring elements present in conditions of pleural effusion or haemothorax.

METHODS.

Albino Wistar rats were used in all experiments. For intrapleural injection, they were lightly anaesthetized with ether and the skin on the right side of the thorax incised. The

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muscles of the chest wall were divided and injection made with a blunt fine gauge hypodermic needle through an exposed intercostal space. The muscles were replaced, the skin sutured and the animals allowed to recover. Unless specifically stated, absorption rates were studied in normally active animals except for the short initial period of anaesthesia required for injection. Nembutal (Abbott) was used for the experiments in anaesthetized rats and was given intravenously.

Homologous heparinized whole blood or plasma was injected and the plasma proteins were labelled with the blue dye, T1824, and the red cells with P^{32}. Plasma was injected in doses of 6 ml. or 2 ml./kg. body weight to which 4 mg./kg. of dye had been added. Plasma for intrapleural injection was usually obtained a day before injection by guillotining and collecting the blood in a vessel containing dry heparin. After centrifugation it was stored overnight in the refrigerator. Weighed amounts of dye were added and thoroughly dissolved before injection.

When the red cells were not labelled, the haematocrit value and haemoglobin concentration of the injected samples were determined. Haemoglobin estimates were made using an M.R.C. Photometer (Keller). Haematocrit determinations were made by the method used by Courtice and Gunton (1949). Absorption rates of fluid were determined by recovering and measuring the unabsorbed fluid using a Pasteur pipette, the animals being killed at given time intervals. Amounts of whole blood absorbed were found by differences in haematocrit and haemoglobin values of injected and recovered blood. Adherent red cells were recovered by washing the pleural cavity with homologous plasma.

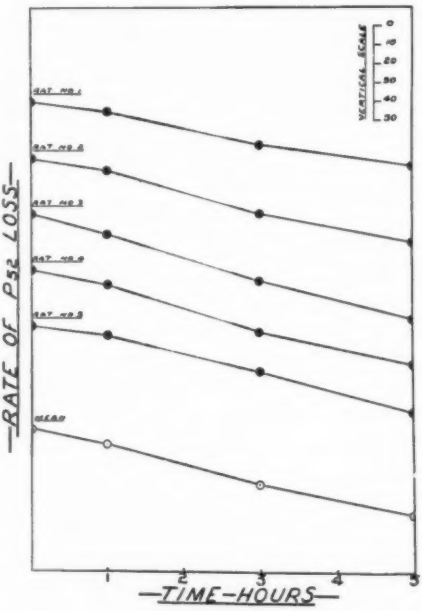

Fig. 1. Showing the percentage loss of P^{32} from rat erythrocytes *in vivo*. Results in 5 individual rats above, and the mean rate of loss below.

P^{32} labelling of red cells was carried out using the technique described by Reeve and Veall (1949). Samples of P^{32} in the form of radioactive phosphate were added to heparinized whole blood at the rate of 5 microcuries to 4 ml. of blood. The whole blood and added radioactive phosphate were incubated for 2 hours at 38° C., the container being rotated slowly throughout to ensure adequate mixing. The red cells were washed in ice-cold saline three times and spun down to remove the excess radioactivity. The red cells with their contained radioactivity were then resuspended in non-radioactive homologous plasma, 2 ml. of plasma being added to the cell content of 4 ml. of blood. This reconstituted blood contained approximately 100,000 counts/ml./minute. Rates of absorption of radioactive red cells were found by estimating the radioactivity in ½ ml. samples of systemic blood taken from the tail vein at various time intervals. A liquid counting Geiger-Muller tube with a volume of 17 ml. was used, the blood samples being made up to volume with phosphate

buffer. Standards were made from original blood diluted 1 in 100 and the radioactivity found in ½ ml. samples of this dilution. Correction factors for the loss of P^{32} from rat erythrocytes *in vivo* were determined after intravenous injection of labelled red cells (Fig. 1). The amount of radioactivity in the plasma was found to be about 1 p.c. of the activity in the red cells. Rat blood volumes were taken as 50 ml./kg. (Sharpe, Culbreth and Klein, 1950) in estimating absorption rates of P^{32} labelled red cells.

The rate of appearance of the T1824 protein complex in the systemic blood was used as an index of plasma protein absorption (Courtice and Simmonds, 1949). The interpretation of dye absorption curves must allow for an unmeasured loss of circulating dye to the tissue proteins. Plasma dye concentrations were determined by the method of Harington, Pochin and Squire (1940) modified for 1 ml. samples of plasma. The optical density of the T1824 butanol extract was measured on a Spekker photoelectric absorptiometer using calibration curves to relate galvanometer deflection to dye concentration (Courtice and Gunton, 1949).

Diaphragmatic paralysis was effected by bilateral avulsion of the phrenic nerves in the lower cervical region. Fluoroscopic examination showed the diaphragm to be moving paradoxically following this surgical intervention. Injections were made in acutely phrenicectomized rats within 48 to 72 hours of phrenicectomy. Animals designated as chronic were injected at varying periods 2 to 4 weeks after paralysis.

To increase respiration by CO_2 inhalation rats were installed in an air-tight cell with inlet and outlet tubes to which 5 p.c. CO_2 in oxygen was delivered at a flow rate of 2 litres/minute.

RESULTS.

The effect of diaphragmatic movement on the absorption of homologous red cells.

Active diaphragmatic movement was eliminated by bilateral phrenic avulsion, decreased by unilateral phrenic avulsion or by anaesthesia and increased by breathing 5 p.c. CO_2 in oxygen.

In initial experiments 6 ml./kg. of whole blood were introduced into the pleural cavity, and the amount remaining was determined in groups of animals at 4, 8 and 16 hours after injection. These experiments were carried out in normal rats and in rats in which one or both phrenic nerves had previously been avulsed. The results of these experiments are given in Table 1. The reduction in the absorption rates in the acutely phrenicectomized groups was significant, $P = <0{\cdot}001$ in the bilateral series and $P = 0{\cdot}05$ to $0{\cdot}01$ in the unilateral series. The chronic group showed a significant increase in their absorption rates above normal, $P = 0{\cdot}05$ to $0{\cdot}01$. After 16 hours practically all fluid had been removed in normal rats, but absorption of red cells was incomplete. A small percentage of cells was still recoverable after 48 hours, the last of the cellular material appearing to adhere particularly to the mediastinal tissues.

The absorption of red cells from smaller volumes of blood was studied by using P^{32}-labelled red cells. Fig. 2 shows the absorption of red cells after 1 ml./kg. of whole blood with labelled red cells was introduced into groups of normal rats and rats with their phrenic nerves avulsed. The normal animals

were further subdivided to study the effects of nembutal anaesthesia and of breathing 5 p.c. CO_2 in oxygen. The figures have been corrected for the loss of P^{32} *in vivo*. An analysis of variance showed significant reductions in absorption rates in the acute phrenicectomized and anaesthetized groups ($P = <0.001$) and significant increases above normal in the chronic phrenicectomized group and in those animals breathing 5 p.c. CO_2 in oxygen.

TABLE 1.

Percentage absorption of red cells after intrapleural injection of 6 ml./kg. homologous whole blood. Mean values shown for groups of 3 rats at each time interval.

	Time after injection		
	4 hours	8 hours	16 hours
Normal	12	30	82
Acute bilateral (1)	7	15	35
Acute unilateral (2)	8	38	45
Chronic bilateral (3)	28	56	69

Analysis of Variance

Source of var.	d.f.	S.S.	M.S.	F	P
Treatments	(3)	(5063)			
Normal v. (1)	1*		2200	24·7	<0.001
Normal v. (2)	1*		512	5·8	0·05–0·01
Normal v. (3)	1*		421	4·7	0·05–0·01
Residual S.S.		(2030)			
Times	2	11627	5814	65·3	<0.001
Interaction†	6	2519	420	4·7	0·05–0·01
Error	24	2146	89		

* Comparisons taken out are non-orthogonal and therefore do not account for the full sum of the squares.

† Interaction sum of squares can be shown to be due to slope differences. Therefore error mean square is used in tests of significance.

The effect of diaphragmatic movement on the absorption of plasma protein from the pleural cavity.

The absorption of plasma protein labelled with the dye, T1824, was determined in normal, acute and chronic phrenicectomized rats at time intervals of $1\frac{1}{2}$, 3 and 5 hours after the intrapleural injection of 6 ml./kg. homologous plasma to which 4 mg./kg. T1824 had been added. In addition the effects of nembutal anaesthesia and of breathing 5 p.c. CO_2 in oxygen were investigated. The results are given in Fig. 3. It is evident that in the acute phrenicectomized animals as well as in the chronic group the absorption of protein is greater than in the normal controls. Similar results were obtained with smaller injections of 2 ml./kg. of plasma containing 4 mg./kg. dye. The analysis of variance of the two absorption rates showed a value of P less than 0·001. Diaphragmatic paralysis in the acute stage was thus seen to have a different effect on the absorption of the protein molecules from that observed with red cells.

The effects of increased respiratory movement with inhalation of 5 p.c. CO_2 on the absorption of plasma proteins and of decreased respiratory movement in anaesthesia are also clearly shown in Fig. 3.

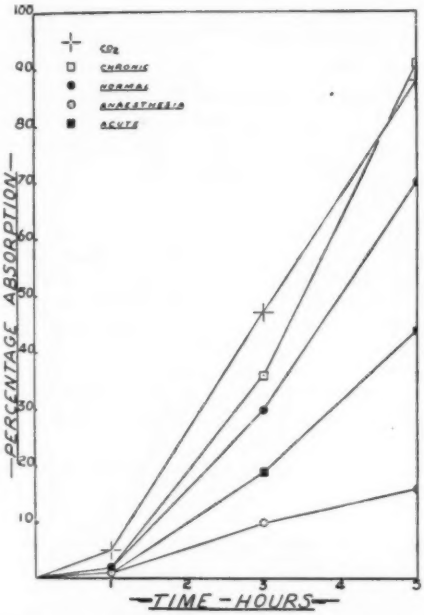

Fig. 2. The rate of absorption of P^{32}-labelled red cells from the pleural cavity of rats after injection of whole blood 1 ml./kg.

+ CO_2 inhalation.
□ Chronic phrenicectomy.
● Normal.
○ Anaesthesia.
■ Acute phrenicectomy.

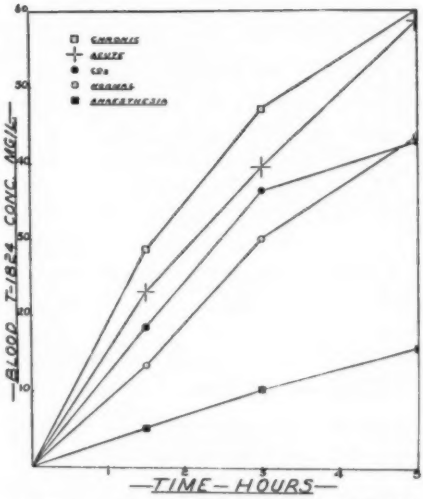

Fig. 3. The rate of absorption of T1824-labelled plasma protein from the pleural cavity of rats after injection, 6 ml./kg.

□ Chronic phrenicectomy.
+ Acute phrenicectomy.
● CO_2 inhalation.
○ Normal.
■ Anaesthesia.

Absorption of P^{32}-labelled red cells in dye-labelled plasma.

A double label experiment was conducted to see if the differential absorption rates between red cells and plasma proteins could be shown when the two elements were injected together. P^{32}-labelled red cells, 1 ml./kg., were resuspended in dyed plasma, 2 mg./kg. in dye in 1 ml./kg. of plasma, and the two labelled components injected into the pleural cavities of normal and acutely phrenicectomized rats. The percentages absorption of the P^{32} red cells and the plasma dye levels in the two groups are shown in Table 2. In this experiment the same slowing in the absorption rate of the P^{32} red cells was reproduced in

those rats with recently paralysed diaphragms, the results agreeing closely with those shown in Fig. 2. The dye concentration in the circulating plasma, which indicated the rate of absorption of the dye-protein complex was, however, slightly higher in the acutely phrenicectomized group. The plasma fraction of the whole blood was not absorbed as rapidly when introduced into the pleural cavity with red cells as when it was injected alone. This was similarly recorded in the peritoneal cavity (Courtice, Harding and Steinbeck, 1953). Although this experiment does not show the same increase in protein absorption in the phrenicectomized animals as occurred in the previous experiments in which plasma alone was injected, the results indicated that diaphragmatic paralysis affected the absorption of cells and protein differently.

TABLE 2.

The percentage absorption of red cells and the dye concentration, mg./litre, in the circulating plasma after the intrapleural injection of 1 ml./kg. labelled red cells and 1 ml./kg. plasma containing 2 mg./kg. dye. Each figure represents the mean of 3 experiments.

	Cells		Plasma	
	Normal	Acute	Normal	Acute
1 hour	2	1	2	2
3 hours	34	14	7·9	8·0
5 hours	64	22	10·8	13·1

Lymphatic pathways.

Following injection of the dyed plasma, the main accumulation occurred in the ventral mediastinal tissues. These were stained a deep blue colour. The mediastinal folds, anterior, retrocardiac, dorsal and ventral were stained by the dyed plasma. Blue dye was constantly present in the costal lymphatics of the parietal pleura, which appeared to drain ventrally to the large sternal lymphatic trunks.

From gravitational influences alone, in the rat, drainage would occur most readily to the ventral regions along the sternum. Accumulation of dye plasma occurred in the subserosal spaces located between the insertions of the diaphragmatic digitations to the costal arch. The sternal lymphatic trunks were filled with blue dye, as were the anterior mediastinal group of lymph nodes. A dorsal route along the vertebral column was constantly blue and this appeared to be draining to the anterior mediastinal group of lymph nodes. The lymphatics beneath the dorsal parietal pleura around the vertebral column and costal insertions similarly contained blue dye. From these observations it appeared that in the rat, as in the rabbit (Courtice and Simmonds, 1949) dye-protein is absorbed into the lymphatics of the mediastinum and the parietal pleura. The visceral pleura covering the lungs rarely showed any staining at all and the diaphragmatic pleura did not appear to be an active absorbing surface.

In view of the fact that the diaphragmatic peritoneum plays such an active part in the lymphatic absorption from the peritoneal cavity, it was decided to attempt to assess the amount of material being absorbed through the diaphragmatic pleura. The transference of absorbed material from the diaphragmatic lymphatics was prevented by ligation of the two sternal lymphatic trunks at the caudal end of the sternum (cf. Courtice and Steinbeck, 1951). In this way the main drainage channels of the diaphragm were blocked without interfering with the lymphatic drainage from the other pleural absorbing surfaces. The results of these experiments in which dye-protein was used to measure absorption are given in Fig. 4 and suggest that the amount of protein absorbed through the diaphragmatic pleura is very small.

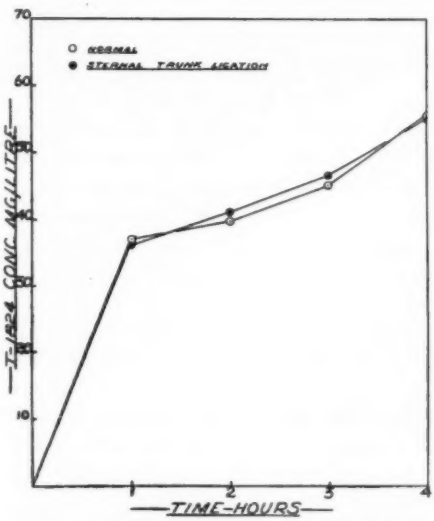

Fig. 4. The effect of sternal trunk ligation near the diaphragm on the circulating plasma dye levels following intrapleural injection, 6 ml./kg.

● Sternal trunk ligation.
O Normal.

The effect of bilateral phrenicectomy on the diaphragm of the rat was found to vary in some of the animals examined. Animals showed varying degrees of atrophic changes from complete atrophy to slight loss and paling of the diaphragmatic muscle tissue (Morris, 1953). Within the first one to two weeks after phrenicectomy the macroscopic atrophic changes were slight, but became progressively more apparent from this point onwards. The injection of fluid at the rate of 6 ml./kg. intrapleurally into acutely phrenicectomized rats produced severe respiratory disturbances in all rats and many died within five hours of injection. The extent to which the intrapleural injection of fluid in these doses interferes with pulmonary ventilation was shown by Davis and Morris (1953).

Post mortem examination of the lungs following the injection of fluid in animals recently phrenicectomized showed degrees of pulmonary collapse that varied with the time the lungs were examined after injection. Five hours after injection the lungs were usually extensively atelectatic and in many cases completely so. The normal rats and those injected two to three weeks after diaphragmatic paralysis were apparently unaffected and *post mortem* there was no evidence of any extensive degree of pulmonary collapse.

DISCUSSION.

Rates of absorption of plasma protein and red cells.

A comparison can be made between the rates of absorption of homologous dye-labelled plasma from the pleural cavities of rats and the absorption rates in rabbits determined by Courtice and Simmonds (1949). These workers found dye concentrations reached values of 13·5 mg./litre 5 hours after injection of homologous plasma, 6 ml./kg., in which 4 mg./kg. dye was dissolved. In rats our results show dye concentrations of the order of 43 mg./litre at this time interval. This points to a more rapid rate of pleural absorption in the rat (cf. Courtice *et al.*, 1953). Dye plasma values are, however, difficult to assess in terms of absolute absorption rates as rates of removal of circulating dye from the plasma are unknown. The absorption of plasma was much more rapid than of whole blood. Plasma, 6 ml./kg., was absorbed in about 8 hours in the normal rat whereas whole blood required about 48 hours for complete removal. Courtice and Simmonds (1949) found a period of about 24 hours was required for the removal of plasma, 6 ml./kg., from the pleural cavity of the rabbit.

Plasma dye curves showed an early steep rise to a maximum at about 5 hours. No initial lag phase was evident when the initial blood sample was taken 1 hour after injection. Red cell absorption curves showed a lag phase for the first hour and then a fairly constant absorption rate until the last few per cent. of cells remained when the absorption slowed. This suggests that the protein molecules more readily pass through the serosal membrane than do the larger red cells in the first phase of absorption into the lymphatics.

The effect of diaphragmatic paralysis on absorption.

The diaphragm, as the chief muscle of respiration in the rat, exerts a profound influence on the pleural dynamics and respiratory functions. Previous work has shown the diaphragmatic paralysis to reduce the tidal air, respiration rate and pulmonary ventilation very significantly in the rat (Davis and Morris, 1953). Diaphragmatic paralysis is seen to affect the absorption of red cells and plasma proteins in two ways in the early period after phrenicectomy. The absorption of red cells is delayed whilst the rate of removal of the plasma proteins is increased.

The process of absorption can be separated into two phases. The first involves the passage of material across the sero-endothelial membrane and into the subserosal lymphatic lacunae. The second phase is the transference of material along the terminal lymphatic capillaries into the main lymphatic trunks and thence to the venous system. The initial phase of absorption is dependent on the presence of a pressure gradient across the mesothelial membrane, created by changes in the volume of the lymphatic lacunae. If the material is con-

sidered to pass through physiological and functional openings in the serosa caused by the stretching and relaxation of the serous membrane then the amount of movement in any absorbing surface will affect the speed of the first phase of absorption. Similarly, if the pressure gradient across the mesothelial membrane is dependent on volume changes of the terminal lacunae the amount of stretching and relaxation of an absorbing surface will be a significant factor in influencing the extent and frequency of development of this pressure gradient. The functional diaphragm would be of importance in stretching the mediastinal folds attached to it and influencing the rate of absorption of material through this site. The pleural surface of the diaphragm itself is probably not an important absorbing surface, nor is the pulmonary pleura in spite of the rhythmic stretching and relaxation of the membrane covering these surfaces.

Cooray (1949) considered the mediastinal folds attached to the diaphragm to be the main areas of pleural absorption and he described in detail the presence of focal aggregations and reticulo-endothelial elements covered by modified mesothelium. These areas are the Kampmeier's foci, with an inherent stickiness through which Cooray considered absorption takes place. In experiments in which graphite particles were used, they were seen to collect in greatest numbers around these foci. If larger particulate matter is absorbed in general more readily through the mediastinal folds and particularly through the Kampmeier's foci, then diaphragmatic paralysis would be expected to reduce the absorption of red cells.

The ease of entry to the lymphatic lacunae will depend on the relative size of the particle to the size of the pore through which it must pass and the pressure gradient to which it is subjected. Plasma proteins having a molecular weight of 69,000-500,000 have an equatorial diameter of 35-38 Ångstrom units (Cohn, Oncley, Strong, Hughes and Armstrong, 1944; Cohn, 1945) compared with 50,000–95,000 Ångstrom units, the equatorial diameter of red cells. The large pore size requisite for the passage of red cells into the lymphatic terminals may be obtained chiefly by stresses exerted by the diaphragm on the specialized areas in the mediastinal folds. Small pore diameters capable of allowing the ready ingress of plasma proteins could be obtained in the comparatively static parietal pleura as well. With the removal of the diaphragmatic influence, increased thoracic girth excursions and stresses on the parietal pleura would favour absorption through the parietal pleura. In these circumstances adequate ventilation depends on the functional ability of the accessory respiratory muscles. The changes occurring in the character of the respiration following phrenicectomy were described by Davis and Morris (1953). Changes in pleural stresses would occur concomitantly with these alterations in the respiration. The increase in thoracic volume required to ventilate the lungs is accomplished by expansion and elevation of the costal arch once active diaphragmatic movements cease.

In the phrenicectomized rat, the chest wall moves extensively during respiration. Fig. 5 shows the changes in the shape of the thorax that follow diaphragmatic paralysis. The phrenicectomized rats become noticeably barrel chested, with widened intercostal spaces and elevated ribs. The characteristic bell shaped thorax of the normal rat caused by the pull of the diaphragm at its costal digitations is absent. The abdomen is pulled in passively with inspiration as the costal arch is elevated and expanded.

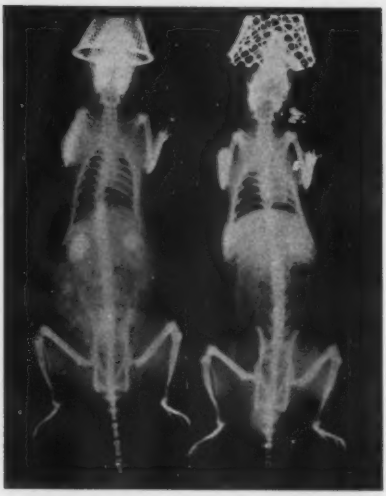

Fig. 5. The change in shape of thoracic cage occurring following phrenicectomy.

Left—Normal rat.
Right—6 weeks after bilateral phrenicectomy.

It seems from the evidence, therefore, that red cells and protein molecules are normally absorbed mainly through the mediastinal pleura, but also to a lesser extent through the parietal pleura. When active diaphragmatic movement ceases, absorption that takes place through the parietal pleura is probably greatly increased because of the increased movement of the intercostal and other accessory muscles of respiration, whereas the absorption through the mediastinal pleura is probably decreased. The fact that in these conditions protein is absorbed more rapidly whereas red cells are absorbed much more slowly than normal suggests that the parietal pleura more readily allows the absorption of protein molecules than of red cells. On the other hand the mediastinal folds, like the diaphragmatic peritoneum, appear readily to admit the larger red cells as well as the smaller protein molecules.

The increase of the absorption rate of red cells to a value near to that of the normal in the chronic phrenicectomized rat is of interest. The acute changes following diaphragmatic paralysis, namely tidal air, respiration rate, pulmonary ventilation and intrapleural pressure have returned to normal in two to three weeks (Davis and Morris, 1953). Not only does the absorption of red cells reach or exceed that in the normal animal, but the already increased absorption of protein in the acutely phrenicectomized animal increases still further as the accessory muscles of respiration become more efficient. This may mean that the red cells can now be more readily absorbed through the parietal pleura. On the other hand the return of the pleural pressures and of the pulmonary

ventilation to normal may enable the mediastinal pleura to function more efficiently as an absorptive surface by allowing a greater movement of the lower part of the lungs and so a greater passive movement of the diaphragm. Custo (1938) also considered return to normal values in the intrapleural pressure to be due to a compensatory increase in accessory muscle tone. Some rats obtain a degree of accessory innervation and return of diaphragmatic function but the exact redistribution of respiratory stresses and reasons for the increased absorption rate are difficult to decide.

Effect of CO_2 inhalation and nembutal anaesthesia on pleural absorption.

Breathing 5 p.c. CO_2 in oxygen had a uniform effect of increasing the absorption rate of both red cells and plasma proteins. Nembutal anaesthesia had a uniformity of effect in the reverse direction. Increased respiratory movements occurring with CO_2 inhalation would increase the phasic changes in the pressure gradient occurring across the serosal membrane. Nembutal anaesthesia would have the opposite effect. All absorbing surfaces would be affected similarly.

The transference of the absorbed material through the larger thoracic lymph trunks is influenced largely by the aspiration effect of intrapleural pressure changes and will depend on the amplitude and frequency of the intrapleural pressure changes. Prinzmetal, Brill and Leake (1931) have shown CO_2 to cause increases in the amplitude of the intrapleural pressure changes. Under nembutal anaesthesia, whilst amplitudes of intrapleural pressure are not smaller, the frequency of these changes is very greatly reduced.

SUMMARY.

The absorption rates from the pleural cavity of plasma protein labelled with the dye T1824 and of red cells labelled with P^{32} have been determined in rats.

Bilateral phrenicectomy in the acute stage decreased the absorption rate of red cells but increased that of plasma protein.

In chronic phrenicectomized animals the absorption rate of red cells returned to normal or exceeded normal while that of protein increased further.

Increased respiratory movement with inhalation of 5 p.c. CO_2 increased the absorption rate of both protein and cells while nembutal anaesthesia had the opposite effect.

The lymphatic absorption of protein and red cells through the different regions of the pleura has been discussed.

Acknowledgments. We are indebted to Dr. W. J. Simmonds for his assistance in the technique of labelling red cells with P^{32}, also to Dr. J. D. Biggers and Mr. P. J. Claringbold for their help in the statistical treatment of the data.

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THE EFFECT OF DIAPHRAGMATIC MOVEMENT ON THE ABSORPTION OF PROTEIN AND OF RED CELLS FROM THE PERITONEAL CAVITY[1]

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The significance of diaphragmatic movement in influencing the absorption of material from the peritoneal cavity has been discussed by previous workers (*cf.* Florey, 1927; Higgins and Graham, 1929; Cunningham, 1926). Material injected into the peritoneal cavity rapidly finds its way to the diaphragmatic absorbing surface through which it passes *via* the lymphatic system to the systemic circulation. Courtice and Steinbeck (1951a) showed that the inclined, pelvis-down position reduced the rate of absorption from the peritoneal cavity, although injected material was still rapidly sucked towards the diaphragm against the gravitational forces. Higgins, Beaver and Lemon (1930), working on dogs, studied the effect of diaphragmatic paralysis on the rate of absorption of graphite particles from the peritoneal cavity. These workers concluded that immediately following diaphragmatic paralysis the absorption of graphite particles from the peritoneal cavity was delayed. Twelve days after paralysis, they found absorption rates to be as rapid as in the normal animal and in cases of long standing paralysis, peritoneal absorption was even faster. The effect of breathing 5 p.c. CO_2 on the rate of lymph flow was reported by Courtice and Steinbeck (1950). These workers used nembutalized cats and cannulated the thoracic and right lymphatic ducts.

Estimates of absorption rates have been largely based on methods which fail to give any exact indication of the actual rate of transference of the injected material from the peritoneal cavity to the systemic circulation. Courtice, Harding and Steinbeck (1953), using large volumes of whole blood, 20 ml./kg., derived quantitative data on the relative rates of peritoneal absorption in rats, guinea-pigs and rabbits. Courtice and Steinbeck (1951b) have also studied the quantitative removal of dye-labelled plasma proteins from the peritoneal cavity of rats.

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[2] George Aitken Pastoral Research Trust Scholar.

The removal of red cells and plasma proteins is effected through the lymphatic radicles of the diaphragm, which drain in turn chiefly to the sternal trunks and thence to the right lymphatic duct. An investigation of the removal of red cells and plasma proteins has been carried out in rats under anaesthesia, breathing CO_2 and with paralysed diaphragms, in an attempt to assess the importance of respiratory movements in peritoneal lymphatic absorption.

Methods.

Albino Wistar and black hooded Lister rats were used in all these experiments on peritoneal absorption. Whole rat blood for injection was obtained by guillotining and collection on to an excess of dry heparin. Plasma free from haemolysis was obtained the previous day and stored overnight in the refrigerator.

In absorption experiments on whole blood, injections were made at the rate of 2 ml./kg. body weight. The cells injected were labelled with P^{32} as a tracer. The method used was that described by Reeve and Veall (1949) and used previously in absorption experiments (Courtice and Morris, 1953). Injected blood contained approximately 100,000 counts/ml./minute and standards were prepared by dilution, the radioactivity in 0·005 ml. samples of injected blood being determined.

Absorption rates were assessed from the amount of radioactivity in ½ ml. sample of systemic blood taken from the tail vein at given time intervals. A correction factor for the loss of P^{32} by rat erythrocytes *in vivo* was used as determined previously (Courtice and Morris, 1953). Blood volumes were taken as 50 ml./kg. body weight (Sharpe, Culbreth and Klein, 1950). The radioactivity of blood samples was determined by using a 17 ml. liquid Geiger-Muller tube, the ½ ml. samples being made up to 17 ml. with phosphate buffer.

T1824-labelled plasma protein was prepared by the technique described by Courtice and Gunton (1949). A weighed amount of T1824, 4 mg./kg., was added to rat plasma, 20 ml./kg., and thoroughly dissolved. As an index of absorption, the dye concentration in the systemic blood was estimated at given time intervals (Courtice and Simmonds, 1949). This did not make any allowance for the amount of dye being removed from the blood stream and being fixed by the tissue proteins, but was considered to give an accurate estimate of relative absorption rates. Absolute values of dye-plasma absorption were derived by differences in amount of T1824 injected and amount recovered in the peritoneal fluid when the rats were killed. Plasma-dye concentrations were determined by the method of Harington, Pochin and Squire (1940) modified for 1 ml. samples of plasma. Optical densities of the butanol extracts were recorded on a Spekker absorptiometer. The injection technique used was described previously (Courtice and Steinbeck, 1951). Rats were anaesthetized only for the period of injection and they recovered and moved about normally in a short while.

Rats subjected to breathing 5 p.c. CO_2 were installed in an air-tight cell as used in pleural absorption experiments (Courtice and Morris, 1953). Five p.c. CO_2 in oxygen was delivered through the cell at a constant flow rate. Anaesthetized animals were injected with nembutal (Abbott) intravenously. Additional injections of anaesthetic were given intramuscularly when animals showed signs of recovery. An initial injection usually maintained anaesthesia at a suitable level for a period of one to three hours.

Diaphragmatic paralysis was effected by a bilateral avulsion of the phrenic nerves in the lower cervical region. Injections were made in acutely phrenicectomized rats within 24 hours of operation and in chronically phrenicectomized rats at least 10 to 14 days following diaphragmatic paralysis.

RESULTS.

Absorption of P^{32}-labelled red cells.

The rate of removal of radioactive red cells, 2 ml./kg., from the peritoneal cavity of the normal rat is shown in Fig. 1. Removal of small amounts of red cells was effected very rapidly. Approximately 72 p.c. of the injected red cells was absorbed within five hours of injection and although the absorption rate levelled off as the fluid was removed from the cellular elements of the blood complete absorption occurred in less than 12 hours.

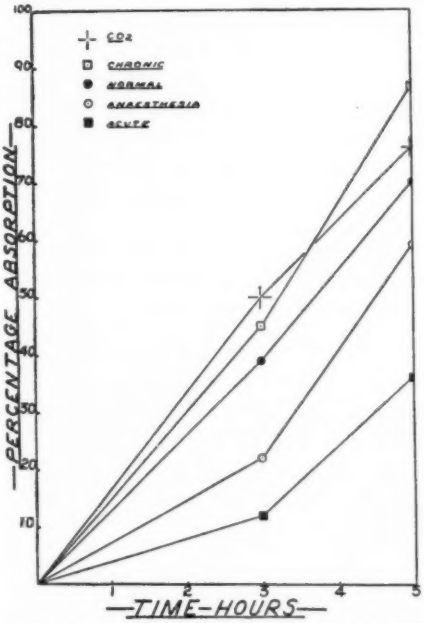

Fig. 1. The absorption of P^{32}-labelled homologous red cells in rats following intraperitoneal injection of whole blood, 2 ml./kg. Each point represents the mean of 6 experiments.

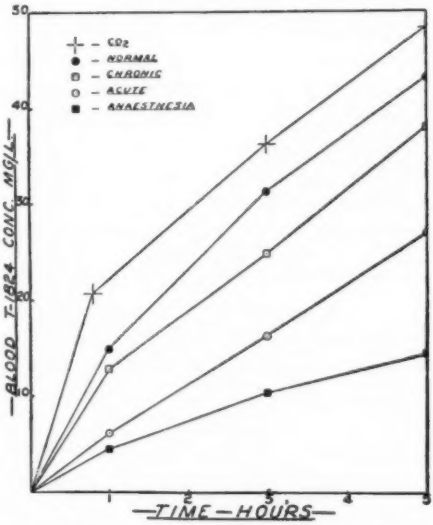

Fig. 2. Plasma dye levels in the circulating blood of the rat following the intraperitoneal injection of homologous plasma, 20 ml./kg. to which T1824 had been added, 4 mg./kg. Each point represents the mean of 6 experiments.

The effect of phrenicectomy. The removal of red cells was very significantly slowed following diaphragmatic paralysis ($P = <0{\cdot}001$) and these rats were capable of absorbing only 36 p.c. of the injected red cells in five hours. The reduction in absorption rate, when active diaphragmatic contractions were eliminated, was about 66 p.c. at the three hour time interval and 50 p.c. at five hours. Fig. 1 shows the absorption rate in acutely phrenicectomized rats.

In rats whose diaphragms were paralysed some three to six weeks prior to injection, the absorption rates had returned to levels as high as or higher than those recorded in the normal series of rats (Fig. 1). In some rats, complete absorption had occurred five hours after injection.

The effect of CO_2 inhalation. Animals breathing 5 p.c. CO_2 in oxygen absorbed red cells at a significantly greater rate than the normal rats breathing air (Fig. 1). From observations, the rats confined in an atmosphere of 5 p.c. CO_2 in oxygen breathed deeply and had rapid respiration rates.

The effect of anaesthesia. Rats subjected to nembutal anaesthesia for the duration of the absorption experiments had significantly slower absorption rates than the normal rats ($P = <0.001$). Respiration rates in the rats in this series varied considerably as the depth of anaesthesia fluctuated. Minimum respiration rates of about 12 to 14/minute were recorded in the deepest stages and maximum rates of 60/minute when anaesthesia became light. After five hours about 57 p.c. of the red cells had been absorbed. It was significant that even with the low respiration rates recorded in many of these anaesthetized rats, absorption rates of red cells were still more rapid than in the unanaesthetized rat with a recent diaphragmatic paralysis. The anaesthetized series had absorbed twice as many red cells in three hours as had the acute phrenicectomy group. These relative absorption rates between anaesthetized and phrenicectomized rats did not pertain in the pleural cavity (*cf.* Courtice and Morris, 1953).

Absorption of dye-labelled protein.

Dye-labelled protein absorption rates were studied in the five groups of rats as with red cells and the relative absorption rates based on plasma dye concentrations in the systemic blood are shown in Fig. 2. In the normal rats plasma dye concentrations reached levels of 31 mg./litre in three hours. About 56 p.c. of the injected T1824-protein complex had been absorbed by these animals in one hour and about 89 p.c. in three hours (Table 1).

TABLE 1.

The percentage absorption of protein from the peritoneal cavity following the intraperitoneal injection of T1824-labelled plasma, 20 ml./kg. Results show means for groups of 6 rats.

	Time after injection	
	1 hour	3 hours
Normal	56	89
Acute phrenicectomy	14	58
Chronic phrenicectomy	34	76
CO_2 inhalation	60	92
Anaesthesia	47	58

The effect of phrenicectomy. Diaphragmatic paralysis 24 hours previous to injection reduced the systemic blood dye levels by more than 50 p.c. and plasma T1824 estimates reached only about 16 mg./litre at three hours (Fig. 2). About

14 p.c. of the injected T1824-protein complex had been absorbed in 1 hour and 58 p.c. in 3 hours (Table 1); these reductions were significant at the 0.001 level. At 5 hours there was an appreciable amount of dye-plasma still recoverable from the peritoneal cavity of these rats.

Animals with diaphragms paralysed 10 to 14 days previously showed absorption rates that were much faster than in those rats with diaphragmatic paralysis of some 24 hours' standing (Fig. 2). These rats had blood dye values approaching those of the normal rats. At 1 hour this group had absorbed about 34 p.c. of the injected plasma protein and about 76 p.c. at 5 hours (Table 1).

The effect of CO_2 inhalation. The group of rats subjected to breathing 5 p.c. CO_2 in oxygen attained higher blood dye levels than the normal series (Fig. 2), and the percentage protein absorption is shown in Table 1. Courtice and Steinbeck (1950) showed very large increases in the rate of thoracic and right lymphatic duct flow when anaesthetized cats were subjected to breathing CO_2 and the effects shown with this experiment were not as great as expected. The respiration of the anaesthetized cat, however, responds very noticeably to the stimulus of CO_2.

The effect of anaesthesia. Rats under nembutal anaesthesia for the duration of the experiment had significantly lower blood dye levels than the normal rats, $P = <0{\cdot}005$ (Fig. 1). Absorption rates of protein were slower than in normal rats, 47 p.c. being absorbed in 1 hour and 58 p.c. in 3 hours (Table 1).

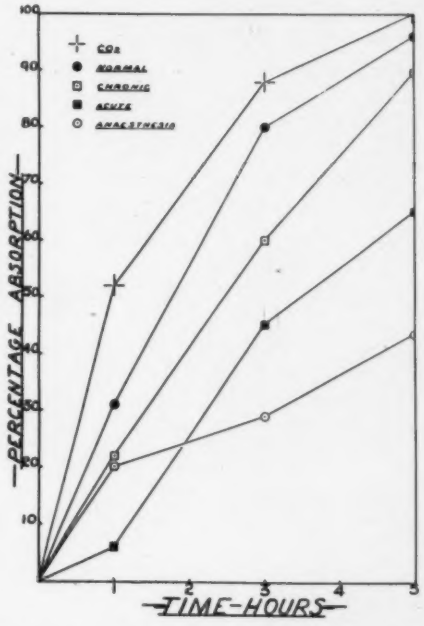

Fig. 3. The absorption of fluid following the intraperitoneal injection of dye-labelled plasma, 20 ml./kg., in the rat. Each point represents the mean of 6 experiments.

The absorption of fluid.

Absorption rates from serous cavities as determined by injection and recovery techniques represent the resultant of two processes occurring after the introduction of the fluid. An effusion of fluid from the vascular system occurs when plasma is injected into the peritoneal cavity. In the interpretation of any figures on fluid absorption rates this must be borne in mind. In this series

of experiments absorption rates were determined by volume differences between injected and recovered fluid. In general there is a close correlation between the rate of dye-protein absorption and the rate of fluid absorption in the five groups of animals studied. Fig. 3 shows the relative absorption rates of fluid between the groups of rats studied. The slowing in the net absorption was significant when the rats were anaesthetized for the period of the experiment and in those animals with recent paralysis of the diaphragm.

Diaphragmatic changes following phrenicectomy.

As the absorbed material must pass through the diaphragmatic peritoneum and into the lymphatics beneath this absorbing surface it was interesting to note the effect of phrenicectomy on the diaphragm. Bilateral cervical phrenicectomy had the effect of immediately eliminating active diaphragmatic movements. Seen under the fluorescent screen, the diaphragm moved paradoxically with respiration and assumed an elevated position in the thorax. Animals deprived of diaphragmatic function were not seemingly affected by intraperitoneal injection of 20 ml./kg. fluid, even a short while after paralysis.

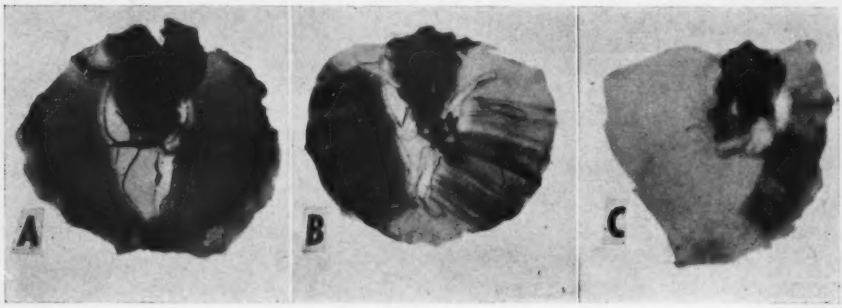

Fig. 4. The loss of muscle tissue in the rat diaphragm following bilateral phrenicectomy. (A) Normal rat. (B) 6 weeks post phrenicectomy. (C) 3 months post phrenicectomy.

Some animals showed indications of return of diaphragmatic function after a variable period of a few weeks. In other rats there was progressive and complete atrophy of the muscle fibres of the diaphragm. Some three to four months after phrenicectomy practically all muscle tissue had disappeared from the diaphragm. Fig. 4 shows the atrophic changes occurring in the rat's diaphragm following bilateral phrenicectomy. There was little gross change observable after loss of phrenic innervation until after the first two to three weeks. The diaphragm then became paler and streaked in appearance. From this point on there was a progressive atrophy of the muscle fibres until the diaphragm became transparent and almost devoid of muscle tissue. Some muscle fibres always remained in the crura.

The reason for return of diaphragmatic function in some of the rats was not clear. An accessory phrenic supply from lower down in the thorax is a possibility. The last intercostal nerve may be involved in the nervous supply to the periphery of the diaphragm near its costal attachments and may supply trophic and motor impulses at least to this regionally limited area.

DISCUSSION.

The extent to which diaphragmatic function influences the removal of injected red cells and plasma proteins from the peritoneal cavity is shown clearly from the results. A reduction in red cell absorption of about 66 p.c. occurs in the stage immediately following phrenicectomy whilst the absorption of plasma proteins is reduced by over 50 p.c. Higgins, Beaver and Lemon (1930), working with dogs, noted an apparent slowing in peritoneal absorption during the first few days following diaphragmatic paralysis. They used a visualization technique and estimated the time taken for injected graphite particles to reach the lymphatics beneath the pleural surface of the diaphragm. In their experiments absorption rates had returned to normal values 12 days after phrenicectomy and became even faster in animals with a longer standing paralysis. It was considered that progressive atrophy of the muscle tissue facilitated the passage of particles through the diaphragm and thus hastened absorption. Courtice and Morris (1953) showed similar conditions to occur in the pleural cavity following phrenicectomy, the absorption rates returning to values as high as, or higher than, in normal rats two to three weeks after diaphragmatic paralysis.

As the permeability of the diaphragm is thought to be of little significance in the absorption of material from the pleural cavity, these workers correlated lymphatic absorption with respiratory function. A return of the respiratory volumes to normal levels is coincident with an increase in the absorption rate. There is seemingly a close correlation between the return of respiratory efficiency and an increase in the peritoneal absorption rate. The atrophy of the muscle tissue of the diaphragm does not seem to offer an adequate explanation for the increased absorption rate which Higgins *et al.* reported to have returned to normal levels 12 days after phrenicectomy.

The changes in the character of the respiration following paralysis of the diaphragm have been described (Davis and Morris, 1953). The increased costal excursions and movement of the abdominal wall would entail a redistribution of stresses around the diaphragm. The possibility of lymphatic channels near the costal insertions of the diaphragm assuming an increased significance in these cases might be considered.

It is of interest to attempt to evaluate the relative significance of the diaphragm in peritoneal and pleural absorption. Courtice and Morris (1953) noted a decrease in the pleural absorption of P^{32}-labelled red cells of about

35 p.c. after phrenicectomy. In the peritoneal cavity the effective rate of reduction is of the order of 66 p.c. In regard to the removal of plasma proteins, phrenicectomy caused no reduction in their rate of removal from the pleural cavity, whilst in the peritoneal cavity the absorption rate was reduced 50 p.c. The function of the diaphragm is consequently considered to play a much more significant rôle in the lymphatic absorption from the peritoneal cavity than from the pleural cavity. The diaphragmatic peritoneum represents the main area through which both red cells and plasma proteins are absorbed, at least when it is actively functioning; this is in contrast to the diaphragmatic pleura which is thought to play a very minor rôle as a lymphatic absorbing surface in the pleural cavity.

SUMMARY.

Some factors affecting the peritoneal absorption of P^{32}-labelled red cells, dye-labelled plasma proteins and fluid in the rat have been investigated.

Diaphragmatic paralysis caused a significant reduction in the lymphatic absorption rate of both red cells and proteins in the early period following phrenicectomy. Absorption rates returned to normal levels or higher two to three weeks following paralysis of the diaphragm.

Anaesthesia caused a significant reduction in the peritoneal absorption rates of both red cells and plasma proteins, whilst breathing 5 p.c. CO_2 in oxygen produced the opposite effect.

The significance of diaphragmatic movement in relation to peritoneal and pleural lymphatic absorption is discussed.

Acknowledgments. My thanks are due to Dr. F. C. Courtice for much helpful advice in this work and assistance in the preparation of the manuscript.

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THE THERMAL DESTRUCTION OF VITAMIN B_1

STUDIES WITH ANEURIN MONONITRATE

by K. T. H. FARRER

(From the Research Laboratories, Kraft Foods Limited, Melbourne).

(Accepted for publication 20th February, 1953.)

Aneurin mononitrate became available in January, 1950, and it was claimed at once that this form of vitamin B_1 was more stable than the previously available synthetic aneurin chloride hydrochloride. The particular application cited was the enrichment of flour (Merck Pamphlet L–1739). Taub, Katz and Katz (1949) found that the mononitrate was somewhat more stable than the hydrochloride in parenteral vitamin B complex and iron solutions, and Macek, Feller and Hanus (1950) claim that the mononitrate is more stable than the hydrochloride in a number of pharmaceutical preparations including solutions and dry products. Bird and Shelton (1950) have also described losses of the mononitrate from various pharmaceuticals but without comparing them with similar figures for the hydrochloride.

More recently Hollenbeck and Obermeyer (1952) have reported that the mononitrate is more stable than the hydrochloride in enriched flour over a storage period of fifteen months at temperatures of 60° F. to 100° F.

The work in this paper compares the thermal destruction of mononitrate and hydrochloride in certain buffer solutions and in yeast extract solutions at 100° C. and of mononitrate and natural vitamins at lower storage temperatures.

Buffer Solutions.

Experimental.

The reagents, assay methods and experimental procedure are similar to those used in earlier papers of this series.

Sodium phosphate solutions. A molar Na_2HPO_4 solution was prepared from A.R. reagent and glass distilled water and freed from heavy metals by the $CaCO_3$ absorption method of Stout and Arnon (1939). When used as a stock solution for the phosphate buffer solutions, the filtered molar solution was just acidified with 6 N HCl (*q.v.*) and the solution was boiled to expel CO_2. The volume was readjusted with twice glass distilled water after the solution had cooled, and the P_2O_5 then checked gravimetrically.

For the citric acid-phosphate buffer solutions the Na_2HPO_4 was not acidified after purification, instead the CO_2 was expelled when the solution was added to the citric acid (*q.v.*).

Citric acid. The purified acid used earlier (Farrer, 1949) was again employed.

6 N Hydrochloric acid. HCl (A.R.) was twice distilled from an all-glass still.

KOH. A.R. reagent was used.

Twice distilled water. Doubly distilled water was prepared in all-glass apparatus with a "strip action" still and was used in the preparation of all solutions.

Aneurin mononitrate solution. Pure aneurin mononitrate (10 mg.) was dissolved in twice distilled water acidified with a few drops of HNO_3 and diluted to 250 ml. 1 ml. = 40 μg. of aneurin mononitrate.

Method. The Jansen thiochrome method was used as described by Farrer (1945). The thiochrome mononitrate behaves in the same way as the product obtained from free aneurin and the hydrochloride. Fluorescence was read with a Coleman Photo-electric Fluorimeter Model 12A.

Procedure. For the phosphate buffer the volume of stock solution required to give a final concentration of M/15 or M/50 was pipetted into a 200 ml. volumetric flask. 10 ml. of the mononitrate solution was added and the solution was diluted to about 180 ml. The pH was adjusted with 6 N HCl or KOH to the desired value using the glass electrode and the solution made up to volume. It was then boiled under reflux in all-glass equipment and samples withdrawn at regular intervals for aneurin assay. A final pH check completed this part of the experiment.

The McIlvaine citric acid-phosphate buffer solutions were prepared as described earlier (Farrer, 1949) by pipetting the amount of M/10 citric acid solution (Britton, 1932) required for the given pH value into a 200 ml. flask and adding slightly less than the calculated amount of M Na_2HPO_4 (unacidified). The flask was warmed and shaken thoroughly to expel CO_2 (from residual $CaCO_3$ in the purified Na_2HPO_4 solution) and made almost to volume. The pH was then adjusted with the phosphate solution and the solution made up to volume with twice distilled water. The traces of CO_2 in the very small final additions of Na_2HPO_4 were lost on boiling and had no influence on the pH. The experiment was then continued as described above.

Results.

The galvanometer readings are equivalent to $(a-x)$ in the monomolecular reaction equation and the curve $\log_{10}(a-x)$/time is an expression of this equation.

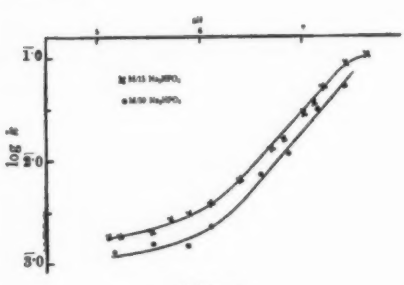


Fig. 1.

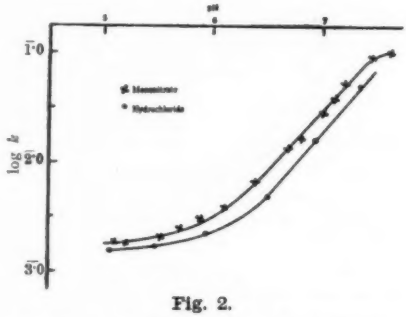


Fig. 2.

Fig. 1. pH/log k curves for destruction of aneurin mononitrate in phosphate buffer solutions at 100° C.

Fig. 2. pH/log k curves for aneurin salts in M/15 Na_2HPO_4 buffer solutions at 100° C.

In Fig. 1 are plotted the pH/log k curves for aneurin mononitrate in M/15 and M/50 phosphate solutions at 100° C. These are compared with those obtained earlier for aneurin hydrochloride (Farrer 1945 and 1947) in Figs. 2 and 3.[1].

[1] It has been found repeatedly that k is reproducible under given conditions. There was therefore no point in repeating in full earlier experiments with the hydrochloride when the results were already available for comparison.

At once it is apparent that the two sets of curves are of the same general shape, i.e., the effect of pH is the same in each case. It is further seen that the mononitrate is rather less stable in phosphate buffer solutions than the hydrochloride.

There is also a concentration effect in that the mononitrate is destroyed less rapidly in the more dilute buffer solution. This appears to be more regular than with the hydrochloride as the effect is seen over the whole pH range studied. However, at pH 5 the difference is greater with the hydrochloride.

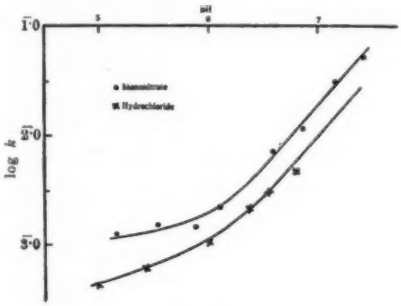


Fig. 3.

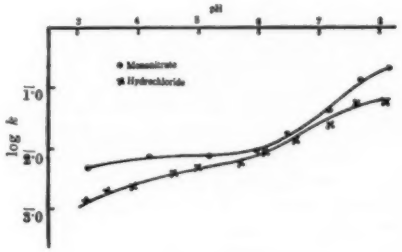


Fig. 4.

Fig. 3. pH/log k curves for aneurin salts in M/50 Na_2HPO_4 buffer solutions at 100° C.

Fig. 4. pH/log k curves for destruction of aneurin salts in phosphate-citric acid buffer solutions at 100° C.

Fig. 4 shows a similar curve for the destruction of the mononitrate in a citric acid-phosphate buffer compared with one obtained earlier (Farrer 1945) for the hydrochloride. Here again there is the same pH effect as was described for the hydrochloride, i.e. there are points of inflexion corresponding with the points of inflexion in the actual buffer curves and the changes in the anionic "species." However, the two curves are not parallel. They are closest at about pH 6·5 and diverge as the pH increases and decreases.

Yeast Extract Solutions.

Yeast extract is prepared by the vacuum concentration of yeast autolysate after the empty yeast cells have been removed centrifugally. It consists essentially of peptones, amino acids, sodium chloride, potassium phosphate and the other yeast minerals and is a potent source of the vitamin B complex including vitamin B_1.

When yeast autolyses the naturally occurring co-carboxylase is hydrolysed to free aneurin (Farrer, 1946) which would then be determined in any assay procedure used to determine any added mononitrate. In these studies a bakers'

yeast extract containing only 5 μg. of aneurin per gm. was used and it was considered that the addition of 150 μg. of mononitrate per gm. of extract would so "swamp" the natural vitamin, which would then be only approximately 5 p.c. of the total, that the behaviour studied would, within experimental error, be the behaviour of the mononitrate.

Experimental.

Procedure. 1·5 gm. of a bakers' yeast extract (5·6 μg. aneurin/mg.) were dissolved in water; 150 μg. of aneurin mononitrate were added and the volume made up almost to 100 ml.

The pH of the solution was measured with the glass electrode and adjusted with acid or alkali to the value shown before making up to volume. The pH of the cooled solution remaining at the end of each experiment was measured. In all cases the shift recorded was within 0·05 pH units.

The solution was refluxed and samples withdrawn at stated time intervals. These were cooled immediately and 5 ml. made up to 100 ml. for assay. As yeast extract is almost completely water soluble and the aneurin mononitrate directly determinable with the thiochrome assay, no preliminary enzyme hydrolysis was necessary.

Assay. The method used consisted of the preliminary washing of an HCl acidified aliquot of a solution of 1 gm. of the extract in 100 ml. of water with an equal volume of water-saturated BuOH (10 ml. of yeast extract solution, 1 ml. of conc. HCl and 10 ml. of BuOH). The washed aqueous layer was made up to 100 ml. and assayed in triplicate at two levels with duplicate blanks. 0·5 p.c. $K_3Fe(CN)_6$ was used in the test solution. A standard solution was assayed concurrently at two levels so that a common zero five point assay results, the aneurin in the yeast extract being calculated by a comparison of the slopes of the two linear curves.

Similar experiments were carried out with aneurin hydrochloride added to a sample of the same yeast extract.

Results.

The results obtained were analysed in the same way as has been usual in this work. In Fig. 5 log k has been plotted against pH. The curve obtained is typical of those obtained with yeast extract solutions (Farrer, unpublished work) and it is very evident that there is no difference in behaviour between hydrochloride and mononitrate.

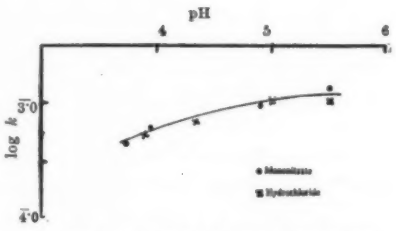


Fig. 5. pH/log k curve for destruction of aneurin salts in yeast extract solutions at 100° C.

Thermal Losses on Storage.

The two food products studied were yeast extract and processed cheese. The same principle of "swamping" the naturally occurring aneurin with added mononitrate was again adopted. Thus to a bakers' yeast extract containing 5 μg. of aneurin per gm. were added 100 μg. of mononitrate per gm.

Bulk cheese contains something of the order of 1 μg./gm. of naturally occurring aneurin and sufficient mononitrate was added to provide at least 20 μg./gm. so that again the aneurin already present would represent only 5 p.c. or less of the total.

Experimental.

Cheese. 2 gm. of aneurin mononitrate (Merck) were added in solution to an experimental 180 lb. batch of processed cheese. After processing according to normal factory practice, a sample of hot cheese was taken direct from the kettle and filled into the bottom halves of a number of petri dishes. The top halves were then forced on and the samples, when cold, were well sealed against loss of moisture at the higher storage temperatures. They were used at 64° C. and 49° C.

Several dozen 8 oz. cartons were taken from the filling machine and stored as such at the lower temperatures (21°, 30°, 37° C.).

The unstored cheese was found to contain 24·1 μg./gm. of aneurin so that, assuming a natural vitamin figure of 1 μg./gm., rather more than 96 p.c. of the total aneurin present was in the form of mononitrate. The pH was 6·14.

Yeast extract. Bakers' yeast extract containing 5μg. of aneurin/gm. was heated in a boiling water bath. When liquid, aneurin mononitrate (100 μg./gm. dissolved in water) was incorporated by thorough stirring. The liquid reinforced extract was filled into 2 oz. screw-capped opal glass jars and allowed to cool overnight. Aneurin determined by the thiochrome assay was 107 μg./gm. and the pH was 5·68.

The results are compared with previously unpublished data obtained in the same way some years ago for the retention of natural aneurin in yeast extract on storage at similar temperatures.

Procedure. Samples were stored at 64–65°, 49°, 43° (44° for cheese), 37°, 30° and 21° C. in incubators. The two series were not done concurrently. Six samples were stored at each temperature and one was withdrawn for assay at the intervals shown below.

Storage Temp. (°C.)	*Sampled*
64°, 65°	daily
49°	every 3 or 4 days
43°, 44°	weekly
37°	every 3 weeks
30°	" " "
21°	" " "

Aneurin assay. Aneurin was determined by the thiochrome method. The procedure of Houston, Kon and Thompson (1940) was used for cheese but with only 4 gm. of cheese instead of 10 gm. because of the much greater vitamin concentration in these samples.

As yeast extract is almost completely water soluble and the aneurin mononitrate directly determinable with the thiochrome assay, no preliminary enzyme hydrolysis was necessary. The assay procedure was as already described for yeast extract solutions.

Results.

Figs. 6, 7, 8 and 9 are vitamin retention curves for cheese and yeast extract. The monomolecular reaction equation has been applied to the results as has been the case in previous work. k_S the velocity coefficient, where t is measured in weeks not minutes (Farrer, 1950), has been calculated for each storage temperature.

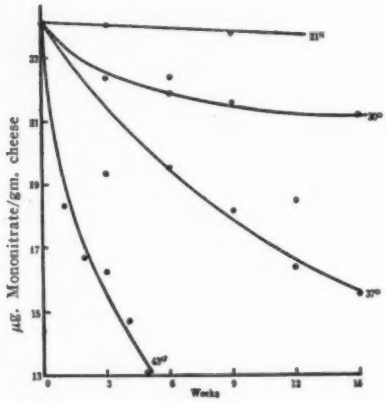
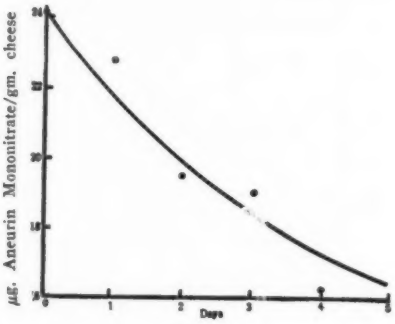


Fig. 6.



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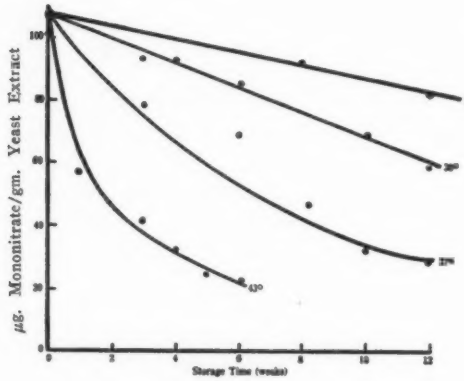

Fig. 8.

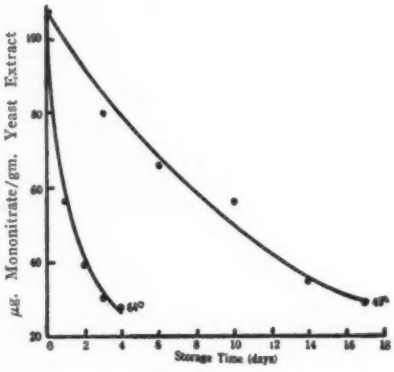

Fig. 9.

Fig. 6. Retention of aneurin mononitrate in stored processed cheese.
Fig. 7. Retention of aneurin mononitrate in processed cheese at 63° C.
Fig. 8. Retention of aneurin mononitrate in stored yeast extract.
Fig. 9. Retention of aneurin mononitrate in stored yeast extract.

The values of log k_S have been plotted against $1/T$ (where temperature is measured in degrees Absolute) in Figs. 10 and 11. These are the curves for the Arrhenius equation, $ln\ k_S = I - E/RT$, which relates velocity coefficient and temperature (I, E and R are constants).

In Fig. 10 the values obtained for cheese have been compared with a similar curve obtained earlier for the thermal destruction of the naturally occurring vitamin on the storage of processed cheese (Farrer, 1950). Although the results

are very similar it would appear that the mononitrate is perhaps slightly more stable than the naturally occurring vitamin at all temperatures. The difference is small, however, and the pattern of its behaviour is the same.

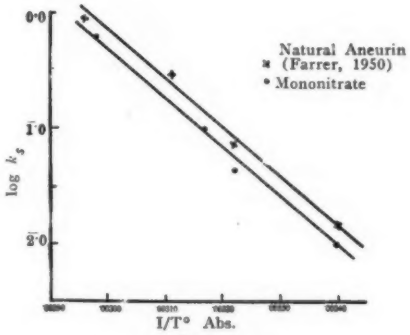

Fig 10.

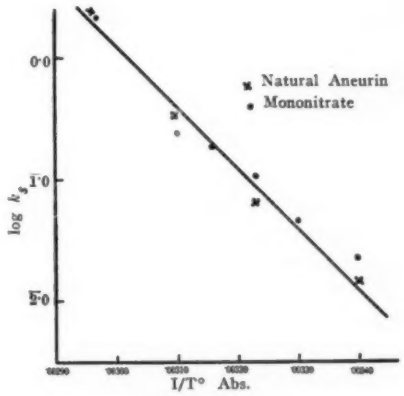

Fig. 11.

Fig. 10. Arrhenius equation curves for destruction of aneurin in processed cheese.
Fig. 11. Arrhenius equation curve for destruction of aneurin in stored yeast extract.

Fig. 11 is the Arrhenius curve for yeast extract and on it values obtained for aneurin mononitrate are compared with those obtained earlier (Farrer, 1947, unpublished) for the natural vitamin. It is readily seen that there is no substantial difference in behaviour between either of the two forms and that, so far as yeast extract is concerned, the mononitrate is not more stable on storage than naturally occurring aneurin. This confirms the observations made in boiling solutions of yeast extract when a direct comparison was made with the hydrochloride. All indications previously have been that the natural vitamins and the hydrochloride behave similarly.

DISCUSSION.

Generally speaking, the behaviour of the mononitrate follows the same pattern as that of the hydrochloride. In the buffer solutions studied the sensitivity to pH, buffer anions and the concentration of buffer salts is very similar for each aneurin salt. In the buffers studied the mononitrate is rather less stable than the hydrochloride.

In yeast extracts and yeast extract solutions each form of aneurin behaves in the same way. One form cannot be said to be more stable than the other. In cheese where the comparison is solely with natural aneurin, the mononitrate is possibly slightly more stable but the difference between the two forms is not great.

The main advantage claimed for the mononitrate was its greater stability in flour under storage conditions and during baking. Certain tests of a preliminary nature were carried out on bread reinforced with both aneurin salts. The results obtained tended to confirm the claims for the greater stability under those conditions of the mononitrate.

Such differences as exist between the two salts can only be attributed to the varying influence on the stability of the molecule, presumably at the C bridge, exerted by the NO_3^- and Cl^- respectively.

SUMMARY.

Using the thiochrome reaction to determine aneurin and reaction kinetics to interpret the results, the thermal stability of aneurin mononitrate and aneurin hydrochloride has been compared in two buffer solutions and yeast extract at 100° C. A comparison has also been obtained for mononitrate and natural aneurin in processed cheese and yeast extract at storage temperatures.

In phosphate and phosphate-citric acid buffer solutions at 100° C. the mononitrate behaves in the same general way as the hydrochloride in its sensitivity to pH, buffer anions and concentration of buffer salts. It is less stable than the hydrochloride.

In yeast extract solutions at 100° C. the two aneurin salts behave in exactly the same way and are destroyed at the same rate.

In processed cheese the mononitrate appears to be slightly more stable than naturally occurring aneurin, but in yeast extract no difference in stability is discernible.

Acknowledgment. The author gladly acknowledges the assistance of Miss J. L. MacEwen and Mr. W. C. J. Hollenberg with the aneurin assays and thanks the Directors of Kraft Foods Limited for permission to publish this paper.

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THE VISCOMETRIC BEHAVIOUR OF A MUCOPROTEIN ISOLATED FROM HUMAN URINE

by C. C. CURTAIN[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 20th January, 1953.)

Since the isolation from urine of a mucoprotein inhibitor of influenza virus haemagglutination (Tamm and Horsfall, 1950; Ada and Gottschalk, 1952) several studies have been carried out on its biological, chemical and physical properties. (Tamm and Horsfall, 1952; Burnet, 1952; Perlmann, Tamm and Horsfall, 1952; Gottschalk, 1952; Odin, 1952).

This mucoprotein is the first inhibitor of influenza virus haemagglutination to be isolated in a state which by electrophoretic and ultracentrifugal criteria (Tamm and Horsfall, 1952) approaches homogeneity. This and its availability in relatively large amounts at small cost enable physical and chemical studies to be made with the aim of increasing our understanding of the interaction between virus, inhibitor and cellular receptor. Knowledge of the properties of this inhibitor may also be of assistance in the purification of inhibitors from other sources.

One of the most striking physical properties of the urinary inhibitor is the high viscosity of its solutions, even at low solute concentrations. Some viscometric studies carried out by Tamm and Horsfall indicated that this effect was due to the asymmetry of the molecule which had an axial ratio of 100:1. The calculation of this value, however, did not take into account the effects of pH and ionic strength on viscosity or the non-Newtonian behaviour which solutions of such an asymmetric molecule would show. It was decided therefore to extend the work of Tamm and Horsfall to include consideration of these factors.

Materials and Methods.

Buffer and salt solutions.

All solutions were made by dissolving analytical reagent grade salts in distilled water. The normalities of calcium and magnesium chloride solutions were checked argentimetrically.

The following stock solutions of buffers were made up and diluted for use at the desired ionic strength.

pH 3·0.	0·8 M glycine, 0·8 M NaCl, 0·2 M HCl.
pH 4·0.	0·8 M acetic acid, 0·2 M sodium acetate.
pH 5·0.	0·3 M acetic acid, 0·7 M sodium acetate.

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra, Australia.

pH 5·89.		0·05 M acetic acid, 0·95 M sodium acetate.
pH 6·0.		0·5 M KH_2PO_4, 0·56 M KOH.
pH 7·0.		0·45 M KH_2PO_4, 0·27 M KOH.
pH 8·0.	(a)	0·5 M KH_2PO_4, 0·47 M KOH.
	(b)	0·56 M sodium borate, 0·44 M HCl.
pH 9·0.		0·85 M sodium borate, 0·15 M HCl.
pH 10·0.		0·62 M glycine, 0·62 M NaCl, 0·38 M NaOH.
pH 11·0.		0·51 M glycine, 0·51 M NaCl, 0·49 M NaOH.
pH 12·0.		0·45 M glycine, 0·45 M NaCl, 0·55 M NaOH.

Receptor destroying enzyme. A purified preparation of the receptor destroying enzyme of *Vibrio cholerae* (RDE) (Ada and French, 1950) was used. The activity of this preparation was determined by the method of Burnet and Stone (1947).

Influenza virus. The classical strain of Lee virus was used in the form of a suspension purified from infected allantoic fluid by adsorption and elution from red cells followed by high speed centrifugation. Details have been previously given by Gottschalk and Perry (1951). The activity of the preparations was titrated by the method of Stone (1951).

"Indicator" virus was prepared by heating the Lee preparation for thirty minutes at 56° C. Destruction of enzymic activity was checked by the method of Stone (1951).

Urinary inhibitor (U.I.) was prepared by the method of Ada and Gottschalk (1952). The biological properties of these preparations were described by Burnet (1952). In the experiments to be described below the method was modified in that the final inhibitor solution was dialysed exhaustively against several changes of excess distilled water and then clarified by centrifugation at 40,000 *g* for 60 minutes. Three layers formed in the centrifuge tube, a sediment, a layer of clear solution and a cloudy layer which floated on the surface. The clear solution was kept. The inhibitory activity was determined by the method of Burnet (1948).

RDE treated U.I. was prepared by incubating the two reagents in the proportion of one unit of RDE to 400 units of U.I. in the presence of 0·00025 N $CaCl_2$ at 37° C. for 30 minutes.

After incubation the solution was dialysed against two changes of excess distilled water and centrifuged at 40,000 *g* for 30 minutes.

Lee virus-treated U.I. This was prepared by incubating the virus with the inhibitor in the proportion of one unit of the virus to 2,000 units of the inhibitor for 180 minutes at 37° C. in the presence of 0·002 N KCl. After incubation the solution was dialysed and centrifuged in the same way as RDE treated U.I.

Control solutions of U.I. These were prepared by incubating solutions of U.I. as above with either RDE or Lee virus which had been inactivated by heating at 85° C. for 100 minutes. The control solutions were dialysed and centrifuged as usual.

Preparations of U.I. solutions for viscosity and solubility measurements. The U.I. solutions were dialysed with stirring for 48 hours against two changes of the desired salt or buffer solution. To remove dust and air bubbles the solutions were centrifuged at 3,000 *g* for ten minutes in an angle head laboratory centrifuge at the temperature at which the viscosity measurements were to be made.

Concentration of U.I. in solutions. This was determined by drying the solutions to a constant weight in a 110° C. air oven. Dry weight blank determinations were carried out on the solvents against which each sample of U.I. had been dialysed.

Partial specific volumes. These were determined at various inhibitor concentrations and in the different salt and buffer solutions using a 10 ml. pycnometer.

High speed centrifugation. This was carried out at 2° C. using a "Spinco" Model L ultracentrifuge. The specifications of the No. 30 rotor employed are as follows:

Angle of tubes to axis of rotation	26°
Minimum radius of column of liquid from axis of rotation	5·0 cm.
Mean radius of column of liquid from axis of rotation	7·8 cm.
Maximum radius of column of liquid from axis of rotation	10·5 cm.
Capacity of each tube (12 in all)	38·5 ml.

Viscosity determinations. These were made with British Standard Ostwald capillary viscometers, B.S. Nos. O, 1, and 2. The relevant dimensions of these are given in Table 1.

TABLE 1.

B.S. No.	Length of capillary	Int. dia. of capillary	Capacity of upper bulb	Capacity of lower bulb	Mean head of liquid
0	12	0·038	6·5	8·0	8·0
1	12	0·060	6·5	8·0	8·0
2	10	0·115	5·5	7·0	7·0

All dimensions are in cm., all volumes in ml.

The viscometers were immersed in a water bath capable of maintaining a temperature of either 25° C. or 37·5° C. constant to within 0·01° C. Flow times were obtained by means of a stop watch which could be read to 0·05 sec. The viscometers were calibrated and checked for consistency by measuring the times of flow of water and several sucrose solutions of known concentration. In this manner it was shown that each of the different viscometers gave the same viscosity value for a Newtonian liquid.

All viscosity values are reported as specific dynamic viscosities, the kinematic correction for the dilute U.I. solutions being negligible.

RESULTS.

Partial specific volumes.

These were found for a concentration range of 0·05–0·5 p.c. (w/v) of U.I. Values lying between the limits 0·705 and 0·711 were obtained. Partial specific volumes were the same for salt and buffer solutions as for distilled water. A mean value of 0·707 was used for the subsequent calculations.

Dependence of viscosity on the dimensions of the viscometer.

In Fig. 1 are given the viscosities of distilled water solutions containing different volume fractions of U.I. measured in the three viscometers. The mean velocity gradient, $\bar{\beta}$, for each viscometer was calculated from the equation of Kroepelin (1929) $\bar{\beta} = \frac{8v}{3\pi r^3 t}$ where v is the volume of liquid passing through a capillary of radius r in time t.

It can be seen that the viscosity of U.I. solutions shows a marked dependence on the velocity gradient in the instrument; that is, the solutions show non-Newtonian flow. This condition was also found in the case of the RDE treated,

virus treated and control inhibitor solutions, all of which gave the same viscosity increment in distilled water as the normal inhibitor in the same viscometer.

For the remaining experiments it was decided to use the No. 2 viscometer and to work at volume fractions below 0·002 since from Fig. 1 the velocity gradient/viscosity relation seemed to be horizontal under these conditions.

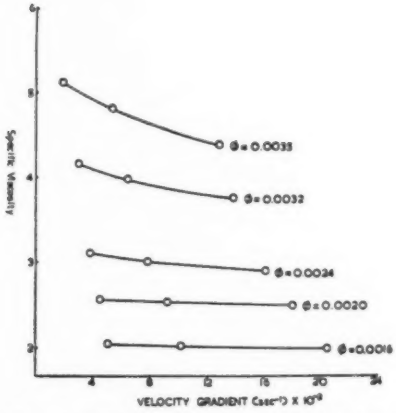

Fig. 1. Variation of viscosity with velocity gradient. Temp. = 25° C. Distilled water solution.

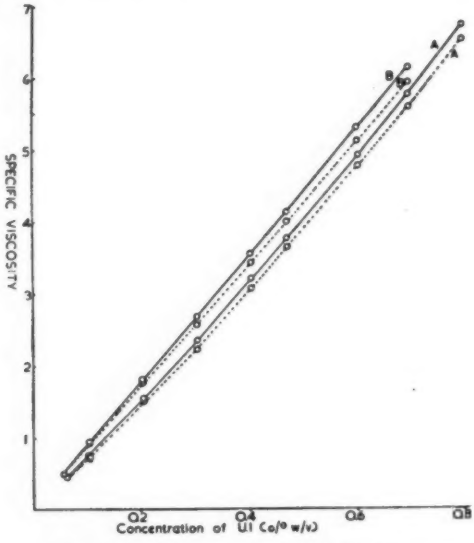

Fig. 2. Variation of viscosity with concentration.

—— measured at 25° C.
---- measured at 37·5° C.
Curves A, solvent 0·05 N KCl.
Curves B, solvent distilled water.

The dependence of viscosity on the concentration of the solutions.

The dependence of the viscosity of U.I. solutions on their concentration is shown in Fig. 2. In 0·05 N KCl at 25 and 37·5° C. a straight line is obtained compared with a curve for distilled water at the same temperatures. It can also be seen that the temperature coefficient of viscosity between the temperatures used is almost zero.

The dependence of viscosity and solubility on pH and ionic strength.

In Fig. 3 are plotted curves giving the variation in viscosity of normal and RDE treated U.I. with ionic strength. It can be seen that the viscosity of U.I. is greatly increased at quite moderate ionic strengths and that this effect is progressively reduced by raising the pH. The viscosity of the RDE treated

U.I. is increased by much lower ionic strengths than the normal. At values over pH 11 (not shown in the Figure) the viscosities of both solutions become identical, dropping markedly, almost to the value for the solvent alone.

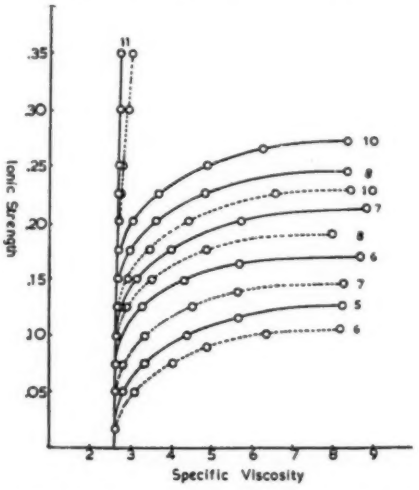

Fig. 3. Variation of viscosity with pH and ionic strength, measured at 25° C.
Volume fraction of U.I. $\phi = 0{\cdot}002$.
—— curve for normal U.I.
---- curve for RDE treated U.I.
The figures at the right-hand end of each curve refer to the pH value.

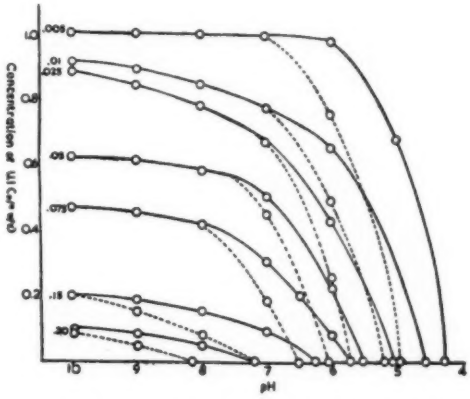

Fig. 4. Variation of solubility with pH and ionic strength.
—— Normal U.I.
---- RDE treated U.I.
The figures at the left-hand end of the curves refer to ionic strength.

Solubilities were determined by centrifuging the samples after dialysis against the desired salt or buffer solution at 2,000 *g* in the ultracentrifuge. The inhibitory activity of each supernatant was determined and the U.I. concentration was found by dry weight. In all cases it was found that the inhibitory activity was proportional to the dry weight concentration of U.I. The variation of solubility with pH and ionic strength is shown in Fig. 4. It can be seen that the solubility of the normal and treated inhibitors follows the trend found with viscosity. The solubility of the RDE treated inhibitor is reduced by much lower salt concentrations and at higher pH values than the normal. In the complete absence of salt it was possible to obtain inhibitor concentrations of up to 4 p.c. by pressure dialysis. These solutions were extremely viscous, but gel formation did not occur.

At ionic strengths above 0·30 both treated and normal U.I. were insoluble except at pH values above 10.

The nature of the precipitates obtained in these experiments is worthy of comment.

As the pH was decreased and the ionic strength increased, independently or together, the solutions passed through a stage of very high viscosity to thixotropic gel formation and finally to precipitation. The precipitates were at first bulky and flocculent, but at either high ionic strengths ($\mu = 0{\cdot}75$–$1{\cdot}0$), or low pH values (pH 2–3) they could be centrifuged out with little trapped fluid. All these precipitates could be dissolved on neutralization and dialysis, yielding apparently normal inhibitor solutions. Full inhibitory activity was recovered in the case of the untreated inhibitor. U.I. solutions held for any length of time above pH 10 did not show normal inhibitory, solubility or viscosity characteristics after neutralization.

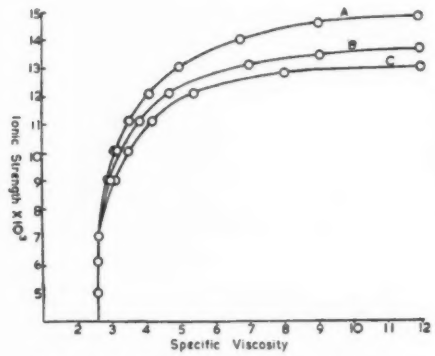

Fig. 5a. Effect of varying concentration of $CaCl_2$ on the viscosity of U.I.
Temp. = 25° C. $\phi = 0{\cdot}002$.
Curve A, normal U.I.
Curve B, Lee virus treated U.I.
Curve C, RDE treated U.I.

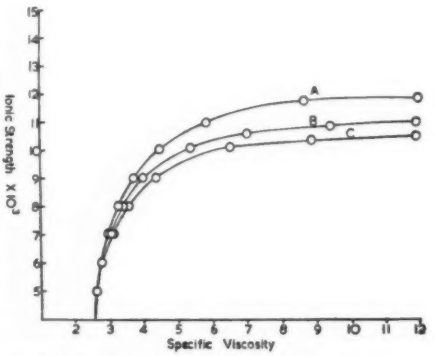

Fig. 5b. Effect of varying concentration of $MgCl_2$ on the viscosity of U.I.
Temp. = 25° C. $\phi = 0{\cdot}002$.
Curve A, normal U.I.
Curve B, Lee virus treated U.I.
Curve C, RDE treated U.I.

The effect of divalent ions on the viscosity and solubility.

In the above experiments buffers containing monovalent sodium and potassium ions were used, and it was thought of interest to examine the effects of some of the divalent cations. In Figs. 5a, b, c, are given the viscosities of normal, virus treated and RDE treated U.I. solutions in the presence of varying ionic strengths of calcium, magnesium and barium chlorides. A striking increase in viscosity is brought about by very low concentrations of these ions, the effect being in the order given. This is most noticeable in the case of the treated inhibitors. At higher ionic strengths gel formation and finally precipitation occur. The gels and precipitates were similar in properties to those obtained in the pH and ionic strength experiments. The treated and normal inhibitors precipitated by Ca and Mg could be redissolved in 0·05 N sodium citrate solu-

tion, or by dialysis against several changes of 0·05 N KCl. In both cases full biological activity of the normal inhibitor was recovered. However, the barium precipitate could not be redissolved by these methods.

Calcium has been shown to play an important rôle in the action of the virus and receptor destroying enzymes on their substrates (Edney, 1949; Burnet and Edney, 1952). It was therefore decided to investigate further its effect on the viscosity of the urinary inhibitor by incubating a U.I. solution at 37·5° C. with RDE or Lee virus (in the proportions given under "Materials") in the presence of 0·005 N $CaCl_2$ in the viscometer and making viscosity measurements at intervals. The results are shown in Fig. 6. Both RDE and virus give a curve tending towards a maximum viscosity with increasing time. The action of RDE gives a greater increase in viscosity than that of the virus, a result to be expected from the data given in Fig. 5. Control determinations carried out with normal inhibitor alone and with "indicator" virus in the presence of 0·005 N $CaCl_2$ both gave the same straight line, also shown in Fig. 6. An interesting feature of this is the slight linear increase in viscosity. Further studies indicated that incubation of sterile solutions of normal U.I. at 37·5° C. in the presence of quite small amounts of electrolytes led to a considerable increase in viscosity and ultimately to gel formation. A viscosity increase occurs on standing at room temperature for longer periods and even in the cold if left for several weeks. U.I. thoroughly dialysed against distilled water never showed a viscosity increase under these conditions.

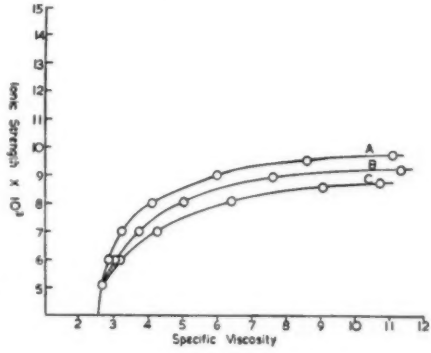

Fig. 5c. Effect of varying concentration of $BaCl_2$ on the viscosity of U.I.
Temp. = 25° C. ϕ = 0·002.
Curve A, normal U.I.
Curve B, Lee virus treated U.I.
Curve C, RDE treated U.I.

The lack of action by "indicator" virus and the shape of the curves given by the increase in viscosity in the presence of active virus suggested that the virus enzyme was involved. In an experiment designed to test this hypothesis RDE was added to U.I. solutions in the viscometer and in a flask also held at 37·5° C. Simultaneously with the viscosity determinations samples were removed from the flask for inhibitor titration and diluted 1/10 in 6·6 M urea solution to stop the enzymic reaction (and to destroy the haemagglutinin of U.I. which would otherwise mask the inhibitor end-point at low titres—Burnet, 1952). In this manner it was shown that the increase in viscosity paralleled the enzymic destruction of the inhibitor.

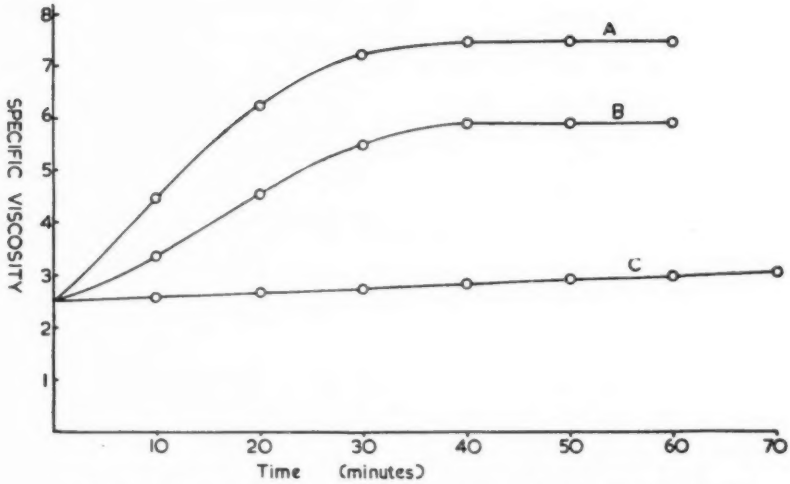

Fig. 6. Effect of incubation with Lee virus and RDE on the viscosity of U.I.

Curve A, with RDE.
Curve B, with Lee virus.
Curve C, control preparations.
Volume fraction of U.I. = 0·0018.

The nature of the anion had little effect on the viscosity or solubility of the normal and treated inhibitor. The points given in Fig. 3 were determined using all the buffers described under "Materials." Identical values for viscosity were obtained with the overlapping acetate, phosphate and borate buffers. This lack of anion effect was confirmed by comparing the effect of varying ionic strengths of K_2SO_4 and KCl. The viscosity and solubility curves for each salt were identical, and fell between those obtained with the pH 6 and 7 buffers.

Results obtained with control U.I.

In a representative number of the experiments reported control U.I. solutions were used in addition to the normal and treated inhibitors. In all cases the control solutions gave identical results to the normal.

Calculation of the axial ratio.

From the viscosities obtained from the straight line portion of Curve A in Fig. 2 a value of the viscosity increment ν was found using the Einstein equation

$$\nu = \frac{1}{\phi}\left(\frac{\eta}{\eta_o} - 1\right) \text{ where } \left(\frac{\eta}{\eta_o} - 1\right)$$

is the specific viscosity and ϕ the volume fraction of the solution occupied by the solute.

A value of 1260 was found for ν. Substitution of this in the Simha equation (Simha, 1940)

$$\nu = \frac{J^2}{15\ (\log 2J - {}^3/_2)} + \frac{J^2}{15\ (\log 2J + {}^1/_2)} + \frac{14}{15}$$

gave a value for the axial ratio, J, of 150 : 1 assuming the molecule to be an unhydrated ellipsoid of revolution.

DISCUSSION.

The value of the axial ratio found above is in approximate agreement with that obtained by Tamm and Horsfall (1952). However, these values should be treated conservatively since the non-Newtonian characteristics of U.I. solutions make it difficult to obtain reliable values for the viscosity increment in the capillary viscometers used both in these experiments and those reported by Tamm and Horsfall. In the study of asymmetric molecules it is desirable that the viscosities of their solutions should be obtained under conditions where the viscometer velocity gradients are accurately known and that the viscosity versus velocity gradient curve be extrapolated to the zero value of the latter. This gives the viscosity increment, ν_0, for random distribution which is more readily interpreted than ν_∞ the viscosity increment for infinite velocity gradient. It is impossible to do this with a capillary viscometer, the velocity gradient of which cannot be accurately defined. The most suitable instrument for these measurements would be a concentric cylinder viscometer of the Couette type, which, however, was not available.

Another difficulty in the way of calculating a value for the axial ratio of U.I. is the effect of ionic strength and pH upon the solutions. These are contrary to the electroviscous effects observed with most proteins. It may be possible to explain these differences on the basis of micelle and gel structure formation influenced by electrostatic forces. The molecules of U.I. have a low isoelectric point (approx. 3·5, Tamm and Horsfall, 1950) and lowering the pH towards this discharges them and decreases the coulombic forces of repulsion between them. According to the theory of Verwey and Overbeek (1948) the latter are balanced by the London van der Waals forces of attraction. The work of Coulson and Davies (1952) suggests that in extended molecules the energy of interaction varies as a power of the length and that the dispersion forces show quite marked directional properties. It is conceivable, therefore, that with molecules the size and shape of U.I. these forces could lead to the formation of orientated micelles and possibly to a gel structure on cancellation of the opposing Coulomb forces. Complete discharge of the molecules would allow the micelles to coalesce into a precipitate.

The increase in viscosity with increasing ionic strength may be explained by supposing that the electrostatic forces between the molecules are influenced by

cation binding, in analogy with the behaviour of myosin (Szent-Györgyi, 1951). The binding of positive ions tends to reduce the negative charge of the molecules facilitating micelle or structural gel formation and so increasing the viscosity. The divalent ions, as in the case of many other proteins, are very strongly bound. This accounts for the marked effect they have on the viscosity and solubility.

It has been shown (Perlmann, Tamm and Horsfall, 1952; Pye, 1953) that treatment of U.I. with RDE or virus leads to an increase in isoelectric point. Thus the treated inhibitors need less hydrogen ions to discharge them to the point of micelle formation explaining why these are more viscous and less soluble at higher pH values than the normal. This also explains the increased effect of cations on the treated inhibitors. At the same pH their net negative charge is lower than that of normal U.I. and so they need to bind fewer cations to be discharged sufficiently to form micelles. Hence the increase in viscosity observed after virus and RDE treatment is a purely electrostatic effect.

From these considerations it can be seen that to obtain the axial ratio value for unassociated molecules of U.I. it is desirable to work either with distilled water solutions or at fairly high pH values and low ionic strengths (pH 8, $\mu = 0{\cdot}05$—see Fig. 3). Fig. 2 shows that in distilled water the viscosity is dependent on the concentration of U.I. in strong solutions. It is necessary, therefore, to use solutions of low concentration (less than 0·2 p.c. (w/v)). The axial ratio value given was calculated from the viscosities of solutions with concentrations in the range below this value.

SUMMARY.

The viscometric behaviour and solubility are described of a mucoprotein inhibitor of influenza virus haemagglutination obtained from human urine. It has been shown that the viscosity and solubility of the inhibitor are markedly affected by variation of the pH and ionic strength of the solution. Increasing the ionic strength and reducing the pH, independently or together, cause an increase in the viscosity and a decrease in the solubility. The solubility and viscosity of the inhibitor after treatment with virus or the receptor destroying enzyme of *V. cholerae* (RDE) are affected at higher pH values and lower ionic strengths than the normal. The alkaline earth cations are several times more effective than monovalent sodium and potassium ions at the same ionic strength. The RDE and virus treated inhibitors are again affected at much lower ionic strengths than the normal.

These effects are explained by assuming that the inhibitor molecules bind cations which neutralize their electrochemical double layers allowing the London van der Waals forces of intermolecular attraction to operate, forming micelles or a gel structure which increases the viscosity. The treated inhibitors have a lower net negative charge than the normal and require fewer cations to discharge them to the point of micelle formation.

Taking into account these effects and the non-Newtonian behaviour of the solutions a value of 150:1 has been calculated for the axial ratio of the inhibitor molecule.

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RHYTHMIC ACTIVITY OF THE ISOLATED MAMMALIAN SPLEEN

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Rhythmic volume changes of the mammalian spleen *in situ* have been a matter of research for some time (Roy, 1881; Schäfer and Moore, 1896; Dale, 1906; Barcroft *et al.*, 1932a; Barcroft and Nisimaru, 1932a; Grindlay *et al.*, 1939a). There is evidence that this phenomenon, although seen mostly under the customary experimental conditions, also occurs in non-anaesthetized animals (Hargis and Mann, 1925; Grindlay *et al.*, 1939b) and may thus have some physiological significance.

Parin (1931) and Barcroft and Nisimaru (1932b) have paid particular attention to the fact—known also to older observers—that the splenic rhythm is often, but not always, associated with rhythmic changes of the arterial blood pressure, a decrease of the spleen volume, as a rule, coinciding with a rise of blood pressure and *vice versa*. It is worth noting that the intestine may also show rhythmic volume changes which, too, are found to be associated with rhythmic blood pressure variations (Barcroft *et al.*, 1932b).

The splenic rhythm was originally attributed to contractions of the spleen. Barcroft and Nisimaru (1932b) assumed accordingly that the mentioned blood pressure changes were due to changes of the circulating blood volume resulting from alternate expressing and storing of splenic blood. The idea of rhythmic spleen contractions, however, had to be abandoned on account of newer findings concerning the blood flow in the splenic vessels. During the rhythmic volume changes the blood flow in the splenic artery and vein shows waves with a slight lag or delay in the veins. (Mertens, 1935; Grindlay *et al.*, 1939b). It is clear that these waves cannot be caused by an activity of the spleen since splenic contractions would affect the blood flow in arteries and veins in opposite ways, that is, by increasing the venous outflow and by decreasing the arterial inflow. These blood flow changes are probably due to rhythmic contractions of the splenic arterioles (Mertens, Grindlay *et al.*).

It thus appears that there is not rhythmic activity of the spleen itself, as was first thought, but rhythmic activity of splenic vessels leading to variations

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council.

of the blood content and to essentially passive volume changes of the organ. With regard to these results it was of some interest to inquire whether the isolated spleen can develop any rhythmic activity at all.

In general, smooth muscle seems to be capable of autonomic rhythmic activity of some degree. In this respect, however, two groups of plain muscular structures can be distinguished: (1) structures which in their normal physiological function contract rhythmically, at least at times (e.g., intestine, bladder, uterus) and (2) structures which normally do not exhibit rhythmic activity (e.g. pilomotors, nictitating membrane, ciliary muscle). It can be said that, as a rule, plain muscle from surviving preparations of the first type develops more or less powerful *spontaneous* rhythm. On the other hand, plain muscle from structures of the second type, with the exception of the blood vessels, shows spontaneous rhythm only exceptionally but can be made to contract rhythmically by suitable chemical or electrical stimuli (Cannon and Rosenblueth, 1932; Eccles and Magladery, 1937). It may be mentioned that the isolated sphincter and dilator of the iris, although they are not known to contract rhythmically during life exhibit slight spontaneous rhythmic tonus changes (Hess *et al.*, 1950; Hess and Koella, 1950). The significance of this rhythm is unknown.

The behaviour of the blood vessels in this respect is remarkable. While there is no evidence that in physiological conditions medium sized vessels perform rhythmic contractions, it is known, on the other hand, that surviving vessels frequently develop a pronounced spontaneous slow rhythmic activity (Meyer, 1913; Full, 1913; Apitz, 1920; Rothlin, 1920a, b, c; Wachholder, 1921; Ducret, 1931; Monnier, 1944; Bürgi, 1944). It is highly improbable that such rhythmic activity could actively assist circulation (Hess, 1916), although Bürgi (1945) has suggested that such a vascular rhythm could serve the peripheral blood distribution.

It may be mentioned in passing that the intermittent contractions of microscopical vessels (metarterioles and precapillary sphincters) observed by Chambers and Zweifach (1944) in some organs (meso-appendix, rat; omentum, dog) and described as vasomotion, have nothing to do with the slow rhythm of isolated bigger vessels. The vasomotion effects local circulatory adjustments.

In considering the rhythmic contractions of isolated vessels one should expect that the spleen, being part of the vascular system, would also exhibit rhythmic activity. However, rhythmic contractions of surviving spleen preparations have never been observed with one exception. Several observers, in fact, made a special point that rhythmic activity of the isolated spleen does not occur (De Boer and Carroll, 1924; Loewe and Faure, 1925; Binet *et al.*, 1927; Saad, 1935). Only Magee (1946) mentioned occasional rhythmic contractions of strips of sheep spleen, but without giving any example and without stating any details.

The experiments described below were undertaken in order to obtain further information regarding this question.

METHODS

Preparations were either obtained from cats killed for this purpose or, as in most cases, they became available from animals previously used for other experiments. Altogether more than 150 strips of cat spleens were examined. The strips were about 3 mm. wide and 20 to 30 mm. long. After having been removed from the body the strips were placed in Tyrode solution and either used immediately or kept in the cold for several days.

In the experiments the preparations were vertically suspended in a Tyrode-filled glass tube, the lower end held by a glass hook near the bottom, the upper end connected with a light aluminium lever above, recording with about twenty times magnification. The preparations were kept stretched by small weights (1 to 2 gm.). The glass tube was immersed in a water bath of constant temperature (about 38° C.). The Tyrode solution within the glass tube contained 0·2 p.c. $NaHCO_3$ and was aerated by a moderate stream of 6 p.c. CO_2/O_2 mixture ensuring a constant pH of 7·4 for indefinite periods (Lippay *et al.*, 1948).

Preparations kept for some time at low temperature were first allowed to assume room temperature before being suspended for the experiment; the temperature of the Tyrode solution was then gradually brought up to the desired level.

Drugs applied were added in solutions of suitable concentration. Before starting the experiments the preparations were first tested as regards their general responsiveness with a small dose of adrenaline (2 to 20 μg.), giving within the Tyrode solution an adrenaline concentration not lower than $1:10^7$. The few preparations which failed to respond were discarded. Some preparations responded to adrenaline concentrations as low as $1:10^8$; but no attempts were made to obtain systematic figures of the effective minimum concentrations of drugs used.

Some experiments were also made on rings of splenic vessels. The arrangement was the same.

RESULTS.

The results of these experiments can be stated as follows:

None of the numerous spleen strips examined has ever shown any sign of spontaneous rhythmic activity, under conditions under which rings of splenic vessels (arteries and veins) frequently exhibited the well known spontaneous rhythm. Thus the negative results of older observers are confirmed on this point.

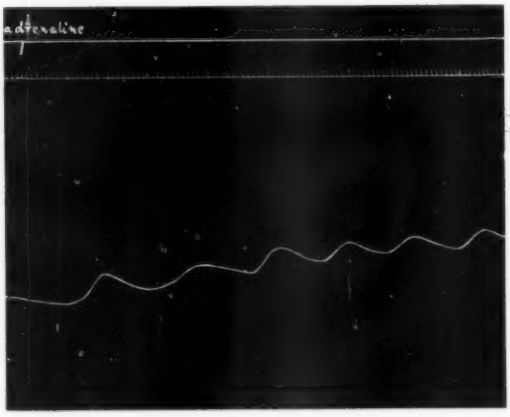

Fig. 1. Strip of a cat's spleen. No spontaneous rhythmic activity. After adrenaline (10 μg.) there appeared a pronounced rhythm superimposed upon a minor tonic contraction (rising base line). Time: 5 seconds.

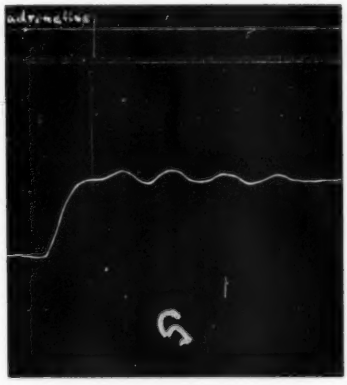

Fig. 2. Strip of a cat's spleen. After adrenaline (30 μg.) there is first a strong contraction, some rhythmic activity of short duration develops at the height of the tonic contraction. Time: 5 seconds.

In about one-third of the preparations, however, adrenaline caused a more or less pronounced rhythmic activity. Very rarely pituitrin and barium chloride proved also effective. Such rhythmic contractions are superimposed on a simultaneous (tonic) contraction (rising base line).

The rhythmic activity induced by adrenaline frequently started as soon as the preparation began to contract; in such cases this rhythm may be the predominant effect, as illustrated in Fig. 1. In other cases there is first a considerable shortening, rhythmic contraction appearing only after this contraction has reached its maximum. Fig. 2 gives an example of this type of response.

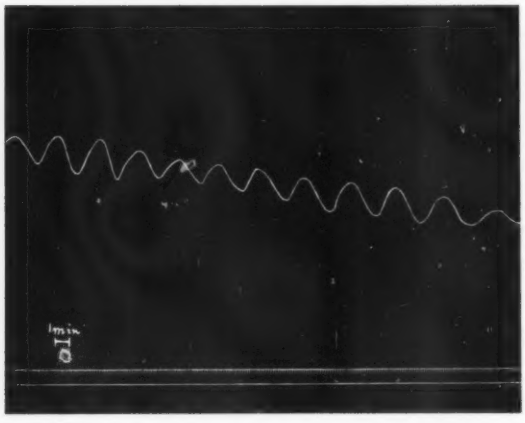

Fig. 3. Strip of a cat's spleen. 20 μg. of adrenaline caused strong rhythmic activity lasting for more than one hour. This rhythm was also present throughout the slow relaxation (falling base line). The fig. shows part of the tracing of this phase. Time: 5 seconds.

As to the duration of this rhythmic activity, it may last only a few minutes or it may go on for more than one hour. The rhythm usually showed periods of one to two minutes. It is of interest that rhythmic activity, if of long duration, may also persist whilst the preparation gradually relaxes (falling base line), as can be seen in Fig. 3.

DISCUSSION.

The results obtained show that the surviving isolated spleen, whilst, unlike blood vessels, utterly incapable of spontaneous rhythmic activity, can, under the influence of drugs, develop rhythmicity in the same way as plain muscular structures which, so far as is known, are not rhythmically active during life. The fact that only a comparatively small proportion of preparations could be induced to contract rhythmically and that this rhythmic activity usually did not last long, seems to indicate that the tendency to automatic rhythm in the plain muscle of the spleen is very low.

The results are in agreement with the finding, mentioned earlier, that the rhythmic volume changes of the spleen *in situ* are apparently not caused by rhythmic activity of the capsule but by rhythmic tonus changes of splenic arterioles resulting in variations of the blood content of the organ. The above results may be taken as additional indirect evidence for the non-existence of rhythmic activity of the plain muscle of the spleen *in situ*.

The splenic rhythm, then, will require reinvestigation as regards its physiological significance. Nothing is known in this respect, although some suggestions, based on the old concept of active splenic contractions, were advanced by Barcroft and Poole (1927). Such a reinvestigation may have to be on a broader basis, taking into account the similar rhythmic volume changes of the intestine (Barcroft *et al.*, 1932b) and the corresponding rhythmic blood flow changes in the hepatic vessels, as observed by Grindlay *et al.* (1941), changes which, owing to existing phase differences, are associated with rhythmic storage and release of blood from the liver and also, in all probability, with rhythmic volume changes. In the light of these facts the splenic rhythm may have to be looked upon not as a phenomenon *sui generis* but as part of a wider pattern of rhythmic circulatory changes within the splanchnic area.

SUMMARY.

The isolated mammalian spleen, unlike the blood vessels, does not exhibit any spontaneous rhythm.

Adrenaline, and very rarely also other agents, can cause such preparations to perform rhythmic contractions. The tendency of the splenic smooth muscle to rhythmic activity is however very low.

This result is in agreement with the fact that the rhythmic volume changes of the spleen *in situ* are not due to rhythmic contractions of the capsule.

It is pointed out that the splenic rhythm during life may be part of a more generalized circulatory rhythmicity within the splanchnic area.

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THE EFFECT OF D.O.C.A. ON DIABETIC KETOSIS

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Diabetic ketosis is associated with a marked increase in adreno-cortical activity (Rose, 1951). It is suggested that part of the insulin resistance and aggravation of the diabetic state which occurs during diabetic ketosis is related to the overactive adrenal cortex with consequent over-production of 11, 17 oxysteroids. These experiments were designed to evaluate the suppressing effect of D.O.C.A. on the increased adrenocortical activity which occurs during diabetic ketosis. Prolonged administration of D.O.C.A. to normal rats can cause adrenocortical atrophy (Ingle, 1937). Moreover, D.O.C.A. has been shown by some workers to prevent the changes in the adrenal cortex which normally occur following stress (Ingle, 1938; Selye, 1942; Woodbury *et al.*, 1950). This suppressive action takes place at the hypothalamic or pituitary but not at the adrenal level (Sayers and Sayers, 1947). However, there are a number of reports indicating the failure of D.O.C.A. to prevent the adrenocortical activation following stress (Sayers and Sayers, 1947). The conflicting results may be related to the type and intensity of the stress and dosage of D.O.C.A. used in the experiment. If D.O.C.A. could in fact suppress the secretory activity of the adrenal cortex an improvement in the diabetic ketotic state might follow.

Method.

Adult male, white, hooded and black rats weighing between 300-320 gm. and bred at the Baker Medical Research Institute were used in these experiments. The animals were made diabetic by the intravenous administration of alloxan at a dose level of 50 mg./kg. They were then placed in individual metabolism cages and fed on a relatively constant diet of cabbage leaves, meat, specially prepared cubes and water *ad libitum*. All animals were tested for diabetes by the determination of blood sugar (Hagedorn and Jensen, 1923). Qualitative tests for glycosuria and urinary ketosis were also carried out.

The diabetic animals were divided into three groups. The first group of diabetic rats received no special treatment. These developed glycosuria and blood sugars of 300–600 mg. p.c. The second group was controlled as well as possible with P.Z. insulin. The insulin injections were commenced 24 hours after alloxan administration and 3–6 units of P.Z. insulin were given daily according to urinary findings. These rats did not develop ketosis although some did show a moderate to severe glycosuria and the blood sugar ranged from

120–300 mg. p.c. The third group of diabetic rats received by subcutaneous injection 2 mg. of D.O.C.A. in 0·2 ml. of oil per day. (All other rats received similar injections of 0·2 ml. of oil per day to act as controls.) The D.O.C.A. and oil injections were commenced 24 hours after alloxan administration.

Ninety-six hours after alloxan administration, the animals were killed by a blow on the head. The adrenals and spleen were immediately removed. The adrenals were placed on filter paper and carefully dissected free from all periadrenal connective tissue and fat, weighed and then placed into 6 p.c. trichloracetic acid if ascorbic acid estimation was required or into alcohol-ether if adrenal cholesterol was to be measured. Adrenal ascorbic acid was measured by the method of Roe and Kuether (1943) and cholesterol by the method of Sackett (1925) modified for tissues. The spleens were dissected free from connective tissue, placed in individual small glass vials and dried in an oven at 80° C. for 12 hours. They were then allowed to cool in a desiccator to room temperature before being weighed.

RESULTS.

Four groups of rats were compared: (i) Normal control; (ii) Uncontrolled alloxan diabetic ketotic; (iii) Alloxan diabetic controlled with P.Z. insulin; (iv) Alloxan diabetic given D.O.C.A.

The results of the first three groups have previously been discussed (Rose, 1951). The main interest of this paper is directed to a comparison between the alloxan diabetic D.O.C.A. treated group with the other three groups.

Table 1 shows the results of wet adrenal weights, adrenal ascorbic acid concentration, adrenal cholesterol concentration and dry splenic weights in each of these groups.

TABLE 1.

		Adrenal weight (mg.)	Adrenal ascorbic acid concentration (mg./100 gm. adrenal)	Adrenal cholesterol concentration gm./100 gm. adrenal)	Dry splenic weight (mg.)
Normal	No. of observations	12	12	12	12
	Mean	39·6	385·3	4·62	266
	Standard Deviation	3·3	45	0·36	44
	Range	36·9–48·3	314–478	4·06–5·15	211–394
Alloxan-diabetic insulin controlled		12	12	12	12
		50·3	405·3	3·66	236
		2·8	68	1·07	81
		46·4–55·1	307–536	1·82–5·12	115–336
Alloxan-diabetic ketotic		24	18	18	12
		60·0	318·3	0·84	100
		4·5	105	0·35	25
		52·7–70·8	154–458	0·39–1·62	70–141
Alloxan-diabetic given D.O.C.A.		21	21	21	12
		50·4	444·7	2·63	176
		5·0	86	1·56	26
		42·9–62·4	306–620	·90–5·32	129–221

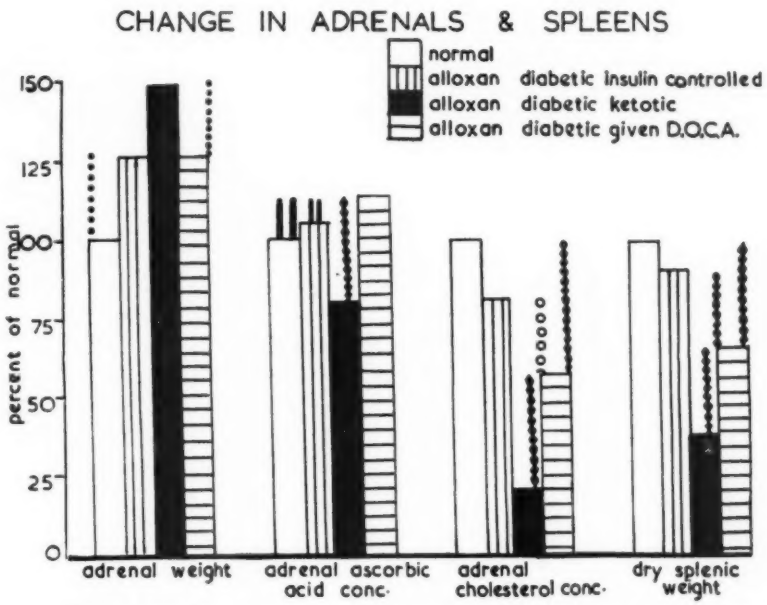

Fig. 1. Graphic comparison of adrenals and spleens in the four groups of experimental animals.

● represents a difference which is statistically significant at the 0·1 p.c. level;

◆ at the 1 p.c. level;

OO at the 5 p.c. level;

❙❙ represents a difference which is not statistically significant.

Fig. 1 summarizes these results and also indicates the comparisons which are statistically significant.

All rats made diabetic had heavier adrenals. This was particularly so in that group which did not receive insulin and hence became ketotic. Treatment with D.O.C.A. reduces the adrenal weight increase to the same extent as insulin control. The mean adrenal ascorbic concentration of the insulin-treated and D.O.C.A.-treated diabetic groups did not differ statistically from the normal. However, the adrenal cholesterol concentration of the D.O.C.A.-treated group, although higher than the diabetic ketotic animals, was statistically less than the normal controls or the insulin-treated diabetic animals. The results for the dry splenic weights follow the same pattern as the cholesterol values. The spleens in the D.O.C.A.-treated group weigh more than those in the diabetic ketotic animals but weigh statistically less than normal controls or insulin-treated diabetic group.

TABLE 2.

Rat Number	Blood sugar concentration in mg. p.c. prior to peanut oil injections	Blood sugar concentration in mg. p.c. 48 hours after onset of peanut oil injections
1	421	314
2	493	430
3	317	436
4	270	297
5	131	217
6	244	315
7	538	468
8	421	266
9	258	356
10	435	216

Table 2 shows the blood sugar response of 10 diabetic ketotic rats 48 hours after treatment with peanut oil. These animals each received a control subcutaneous injection of 0·2 ml. of peanut oil immediately and 24 hours after the first blood sample was taken. The peanut oil injections had no consistent effect on the blood sugar.

TABLE 3.

Rat Number	Blood sugar concentration in mg. p.c. prior to D.O.C.A. injections	Blood sugar concentration in mg. p.c. 48 hours after onset of D.O.C.A. injections
1	421	131
2	300	138
3	345	122
4	401	197
5	430	134
6	408	148
7	442	153
8	431	149
9	362	121
10	324	144

Table 3 shows the blood sugar response of 10 diabetic ketotic rats 48 hours after treatment with D.O.C.A. These animals each received by subcutaneous injection 2 mg. of D.O.C.A. in 0·2 ml. of oil immediately and 24 hours after the first blood sample was taken. The administration of D.O.C.A. caused a fall in the blood sugar.

DISCUSSION.

In this experiment adrenal weight, adrenal cholesterol concentration, adrenal ascorbic acid concentration and dry splenic weights have been used as indices of adrenocortical secretory activity.

There is no significant difference in the adrenal weight and ascorbic acid

concentration between the D.O.C.A.-treated and insulin-controlled groups. However, the adrenal cholesterol concentration and dry splenic weights are significantly less in the D.O.C.A.-treated than in the insulin-controlled animals. These results indicate that there is a greater adrenocortical activity in the D.O.C.A.-treated than in the insulin-controlled group.

The results indicate that D.O.C.A. administration suppresses some but not all the increased adrenocortical activity which is associated with uncontrolled diabetic ketosis. The D.O.C.A.-treated rats still show some increased adrenocortical activity. It is apparent that 2 mg. of D.O.C.A. per day does not entirely prevent the increased adrenocortical activity of diabetic ketosis.

In these experiments administration of D.O.C.A. to diabetic rats resulted in a fall of blood sugar with a partial suppression of the adrenal cortex. These two actions of D.O.C.A. may well be related. It has been shown that small doses of D.O.C.A. increased the sensitivity of the rat to insulin (Cheng and Sayers, 1949), and decreased the glycosuria of the partially depancreatized rat (Ingle, 1948). Improved carbohydrate tolerance has been reported in humans following D.O.C.A. administration (Winnett, 1940; McGavack *et al.*, 1941; Kohler and Fleckenstein, 1942). Zimmerman *et al.* (1951) demonstrated a transient increase in sensitivity to insulin only in those patients who were initially insulin-resistant. In the initially insulin-sensitive patient D.O.C.A. produced either a decrease in insulin sensitivity or no change. A similar "diabetogenic" effect of D.O.C.A. has been demonstrated in the partially depancreatized rat with very large doses of D.O.C.A. (Ingle, 1948).

These conflicting results suggest that D.O.C.A. may influence carbohydrate metabolism in a number of different ways. A consideration of these mechanisms is pertinent to this discussion.

1. D.O.C.A. may inhibit A.C.T.H. production of the pituitary thus decreasing the secretion of 11 oxysteroids by the adrenal cortex.

2. Large doses of D.O.C.A. may act as a stress and increase adreno-cortical activity (Selye, 1948).

3. D.O.C.A. may be converted by the adrenal cortex into a steroid which has an effect on carbohydrate metabolism. This has been demonstrated in isolated adrenal perfusion experiments (Hechter *et al.*, 1951) and in adrenal brei (Hayano *et al.*, 1949; Savard *et al.*, 1950; Haines, 1952). The main product obtained by such experiments is corticosterone.

4. It is generally thought that D.O.C.A. has no direct effect on carbohydrate metabolism. Thus D.O.C.A. given to a fasted adrenalectomized rat does not maintain the blood sugar (Long *et al.*, 1940) or cause the deposition of liver glycogen (Olsen *et al.*, 1944) or protect the animals from insulin sensitivity (Lewis *et al.*, 1940). Similarly in humans D.O.C.A. does not repair the abnormalities of carbohydrate metabolism found in Addison's disease (Thorn *et al.*, 1940). It should, however, be noted that some authors (Sass-Kortsak *et al.*,

1949; Wang and Verzar, 1949) have demonstrated a glycogenetic effect of prolonged D.O.C.A. administration in both muscles and liver of the adrenalectomized rat.

SUMMARY.

There is a marked increase in adrenocortical activity in uncontrolled, alloxan-diabetic, ketotic rats.

D.O.C.A. partially suppresses this increased adrenal activity but not to the same extent as insulin control.

D.O.C.A. administration to diabetic, ketotic rats causes a fall in blood sugar.

The fall in blood sugar may be related to the partial suppression of adrenal activity and consequent decline in production of 11, 17 oxysteroids. The validity of this causal relationship has been discussed.

Acknowledgments. I would like to thank the Baker Medical Research Institute for providing facilities and animals during the course of these experiments. I am grateful to Miss A. Loukes and Miss P. Trewhella for technical assistance; and to Professor Belz for the statistical analysis of these results.

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DETERMINATION OF BLOOD SUGAR WITH ANTHRONE

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(Accepted for publication 2nd January, 1953.)

The use of Dreywood's (1946) anthrone reagent for the quantitative estimation of carbohydrates has been described in the literature by a number of workers (Morris, 1948; Seifter, Dayton, Novic and Muntwyler, 1950; Peterson and Rose, 1951; Zipf and Waldo, 1952). However, to our knowledge, a reliable application of the method to the measurement of blood-sugar has not been published, although Morris (1948) in his original letter to *Science* did mention that this was practicable. The reason for this omission is probably due to the fact that existing methods, in particular the Hagedorn-Jensen (1923) technique, are quite satisfactory and rapid. However, the assay described below has certain advantages which will be outlined.

METHOD.

A sample of blood containing approximately 20–300 μg. of glucose is pipetted into 5·0 ml. of distilled water in a centrifuge tube, 0·2 ml. 0·6N NaOH and 0·2 ml. 10 p.c. $ZnSO_4.7H_2O$ are added and mixed (Somogyi, 1930). After standing for at least 10 minutes the mixture is centrifuged to yield a clear supernatant solution which can, if necessary, be decanted into a clean dry test tube.

Duplicate aliquots of 2·0 ml. of the supernatant liquid are pipetted into a 6″× ¾″ test tube. These are placed in a bath of melting ice, and 4 ml. of 0·2 p.c. anthrone in 95 p.c. H_2SO_4 (as prepared by Morris) is then added by fast flowing pipette and thoroughly mixed with the glucose solution *in the cold.*

A blank using distilled water and standards containing 40 μg. of glucose are also prepared.

The test tubes are then stoppered with one-holed rubber stoppers carrying a thick glass capillary (Van Slyke and Hawkins, 1928) and placed in a boiling water-bath for 7 minutes. At the end of this time, the tubes are rapidly cooled in running water to room temperature, and the colours developed are measured against the blank in a colorimeter or spectrophotometrically at 620 mμ. Since the colour development obeys the Beer-Lambert Law, the glucose concentrations of the samples are immediately determined by simple proportion with the optical density of the standard (40 μg.).

If the anthrone solution is always freshly prepared in exactly the same manner and from the same initial reagents, it may be sufficient to read off the concentration from a standard curve or from a reading-concentration relationship which is determined initially. It may be noted here that the purity of the sulphuric acid used is as critical as the age of the anthrone solution. Since these two factors may have a significant effect on the results, we always use standards in practice.

[1] Assisted by a grant from Nicholas Proprietary Limited.

EXPERIMENTAL.

Preparation of blood filtrates. It was found that most methods of preparation which involved boiling and filtering (Hagedorn and Jensen, 1923) gave unreliable results. Most filter papers appear to contain some water-soluble carbohydrate material, causing a small fluctuating positive error as a result of the extreme sensitivity of the assay. Glass wool as a filtering medium was found to be satisfactory and this should be used in the absence of a centrifuge.

Of all the methods tried, however, centrifuging in the cold is the quickest and most satisfactory.

The time of heating to obtain the maximum colour development is critical. Glucose solutions reach their maximum intensity after 6 minutes, maintain it for a further 2 minutes, and then gradually fade if further heated (see Table 1). When cooled, the colour attained is stable for several hours.

Sucrose solutions attain their maximum colour over the period 4–6 minutes. Hence such solutions do not provide true standards for glucose measurements.

The curve of colour development with heating may, to some extent, depend on the heating apparatus, type of test tubes used, volume heated, intensity of heat source, etc., available. It may be noted that Seifter and co-workers (1950) obtained their maximum colour development after 9–10 minutes heating with different conditions, and it is probably advisable for workers using this method to determine beforehand the curve of colour development for the specific circumstances of their technique.

TABLE 1.

Effect of heating time on colour development.

Time of heating (mins.)	Optical density at 620 mμ. Glucose (40 μg.)		Sucrose (40 μg.)		Glycogen	
1	·045		·306		—	
2	·172		·335		·312	
3	·287		·360		·480	
4	·343		·381	(Max.)	·550	
5	·369		·372		·640	
6	·392		·363		·680	
7	·395	Max.	·352		·681	Max.
8	·390		·336		·686	
9	·375		·324		·685	
10	·370		·305		·650	
11	—		—		·627	

(All figures are averages of duplicate determinations.)

Precision and sensitivity of method. Best results are obtained with aliquots containing about 40–60 μg. glucose. Optical densities of about 0·4 in the centre of the spectro-absorption scale are then obtained and the standard error for such concentrations does not exceed 2 p.c.

2 ml. samples containing as low as 6–10 μg. glucose can be determined with a standard error of about 5 p.c. Thus the method enables the determination of

the glucose concentration of a 0·02 ml. sample of normal blood. In fact, a 0·01 ml. sample can be assayed by using half-quantities of water, sodium hydroxide and zinc sulphate in its preparation.

Effect of anticoagulants. Blood was taken from a rat and 0·1 ml. portions were mixed, in duplicate, with various anticoagulants and with water (to give a control) and then assayed as above.

It is clear from the results which are set out in Table 2 that the anticoagulants added had no effect on the assay.

TABLE 2.

Effect of anticoagulants on anthrone assay.

Sample	Optical density at 620 mμ.
0·1 ml. blood + 5·25 ml. water	·568, ·565
0·1 ml. blood + 0·25 ml. 3·5 p.c. sodium citrate	·564, ·559
0·1 ml. blood + 0·25 ml. 1·1 p.c. sodium fluoride	·569, ·572
0·1 ml. blood + 0·25 ml. heparin	·558, ·559
0·1 ml. blood + 0·25 ml. 1·6 p.c. sodium oxalate	·585, ·563

(5·0 ml. water was also added to each of the four test samples.)

Accuracy of method. Known quantities of glucose were added to blood samples and found to be 100 p.c. recoverable. Hence it is clear that the assay is not inhibited or affected by any factor in the water soluble fraction of blood.

The assay gives, in general, results which are about 20 mg./100 ml. lower than those obtained by the Hagedorn-Jensen method. It is not difficult to show that this is due to over-estimation of the blood-sugar by the latter method which measures other reducing substances. To confirm this the following experiment was carried out: 6 ml. of blood were removed from a rabbit and immediately assayed by both methods. The rest of the blood was divided into two parts to which heparin and oxalate were added respectively. Both portions were then incubated at 37° C. for 24 hours after which time it was expected that glycosis would be complete (Dumazert and Penet, 1939). They were then also assayed by both methods. The results are shown in Table 3.

TABLE 3.

	Anthrone assay mg./100 ml.	Hagedorn-Jensen mg./100 ml.
Initial assay	98·8	127·5
After 24 hours:		
Heparinised blood	3·2	23
Oxalated blood	6·0	19

It would appear, therefore, that the anthrone assay gives a better indication of the true glucose concentration than does the Hagedorn-Jensen procedure.

Determination of glycogen. The same reagent may be used for the determination of glycogen. The method is fully outlined by Seifter (1950) and has one great advantage that it does not require the prior hydrolysis of the glycogen.

It was found that with our heating technique, the maximum colour was attained over the period 6–9 minutes, in the case of glycogen (see Table 1). Hence glucose standards may also be used for this assay.

DISCUSSION.

The anthrone assay for blood sugar is no quicker than existing methods for this determination. Moreover, it requires a special reagent and the use of a spectrophotometer or absorptiometer for the final measurement. The latter is not a serious objection as most laboratories are now equipped with such instruments. The anthrone reagent is easily prepared and can, moreover, be used for a rapid and reliable estimation of glycogen and, in fact, of many other carbohydrates (Morris, 1948).

The main advantages of the method lie in its sensitivity and specificity to carbohydrates. It is sensitive to 6 μg. of glucose and hence as little as 0·01 ml. of blood can be assayed if necessary. This is often an important consideration in modern physiological experiments with very small animals. The method gives a closer approximation to the true glucose concentration than methods measuring all reducing substances, and interference from non-carbohydrate sources is negligible (Seifter, Dayton, Novic and Muntwyler, 1950).

The assay has been used for the past six months in our laboratory and has been found quite satisfactory for routine estimations. It lends itself particularly to large-scale experiments when a great number of blood-sugars need to be determined.

SUMMARY.

A rapid method for the determination of blood-glucose is described.

The method is suitable for the estimation of amounts of glucose between 6 and 100 μg., the precision being greatest for 40–60 μg.

It gives a more accurate measure of blood-glucose concentration than most existing methods and is not affected by the anti-coagulants normally used for blood-preservation.

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THE INFLUENCE OF STEROIDS ON THE CARCINOGENIC ACTION OF 2-ACETYLAMINOFLUORENE

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The incidence and distribution of tumours produced by 2-acetylaminofluorene in rats have been the subject of numerous reports since the original description of the carcinogenic action of this substance by Wilson *et al.* (1941). In experiments where the carcinogen has been fed to animals, there is considerable divergence in recorded results regarding the time of onset and tissue site of tumour formation. However, unanimity does appear to exist on the high incidence of liver damage in rats, especially albinos, fed the substance over a period of time. The existence of sex hormone influences on the tissue distribution and development of tumours has been established by Bielschowsky (1944) and Cantarow *et al.* (1946, 1948) and pituitary and adrenal factors have also been postulated, but the evidence in support of these claims is more tenuous.

Experiments on the direct passage of adrenal cortical hormones through the liver by adrenal transplants into spleen (Read, 1951) have shown that there is little or no inactivation of adrenal steroids by the liver, thus confirming earlier acute experiments (Vogt, 1947). These facts appeared to offer an approach to determining any possible direct or intermediary rôle that adrenal cortical hormones might have on liver damage and hepatic tumour formation. Also some information about the subsequent influence of steroids on distant organs after this immediate passage through the liver was considered to be a likely outcome of the experiment.

MATERIALS AND METHODS.

Adult male and female albino rats (descendants of the Wistar strain) weighing between 120 to 220 gm. were used. Over the past six years, no spontaneous tumours, except two cystadenomas of the breast, have been observed in this colony. The experimental groups were weighed weekly. The diet was the normal routine rat pellet used in this laboratory and consisted of: wheat meal, maize meal, pollard, bran, wheat germ, meat meal, fish meal, skim milk powder, lucerne meal, dried yeast, shark oil, calcium carbonate and salt. To water-moistened and powdered pellets, 4 mg. of 2-acetylaminofluorene per rat per day were added. The substance was dissolved in alcohol first and thoroughly mixed after addition. There were no supplements to this diet and it was fed to the rats six days a week for a

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total period of nine months, after which the animals were sacrificed, one from each group at the same time, over a period of three months.

The 2-acetylaminofluorene had been prepared by standard methods.

Histological staining was haematoxylin and eosin.

Adrenal transplants were done by the method described by Read (1951) and the stilboestrol in one group was given by the subcutaneous implantation of 8 mg. pellets.

RESULTS.

General.

No breast tumours were observed in any of the experimental animals.

In one group with adrenal transplants into spleen and stilboestrol implanted subcutaneously, thirteen of sixteen animals died as soon as 1 p.c. saline in drinking water was replaced by ordinary tap water. The three surviving animals in this group are reported.

As far as weight was concerned, we could not use it as an index of the development of tumours as there were no significant variations in the vast majority of individual rats in any group. In general, there was little gain in weight in all the rats over this quite long period when considerable increases might have been expected. This confirms the experience of Wilson *et al.* (1941).

We observed no metastatic deposits.

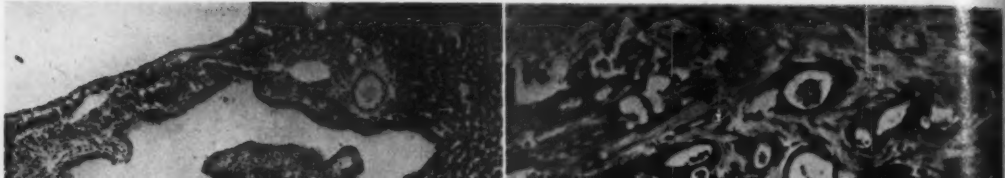
Lung lesions, including inflammatory changes, bronchopneumonia and frank lung abscesses were much more frequent than we have ever encountered before.

TABLE 1.

Eleven animals in each group. All fed 2-A.A.F.

Group and sex	Experimental procedure	Liver					Skin tumours	Ovary tumours
		N	A	B	C	M†		
1. Females	Adrenal transplants into spleen	3	3	5	–	–	1	3
2. Females	Normal controls	2	5	3	1	–	–	–
3. Females	Spayed and adrenal transplants into spleen	–	4	7	–	–	–	–
4. Females	Spayed	–	5	6	–	–	–	–
5.* Females	Spayed + adrenal transplants + stilboestrol implants	–	–	2	1	–	1	–
6. Males	Castrated	–	–	4	–	7	–	–
7. Males	Normal controls	–	2	6	1	2	1	–

* Group 5 consists only of 3 surviving animals. † See text for definition.



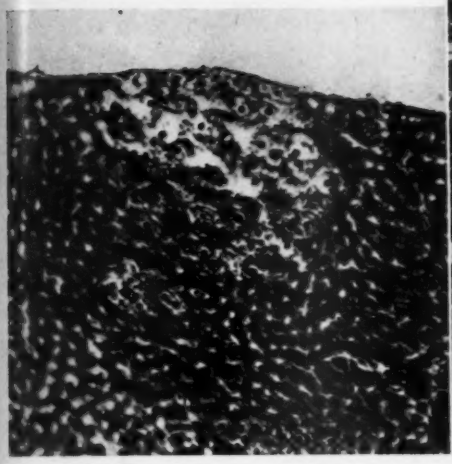


Fig. 1. Liver. A focus of degeneration. Haematoxylin and Eosin. × 150.

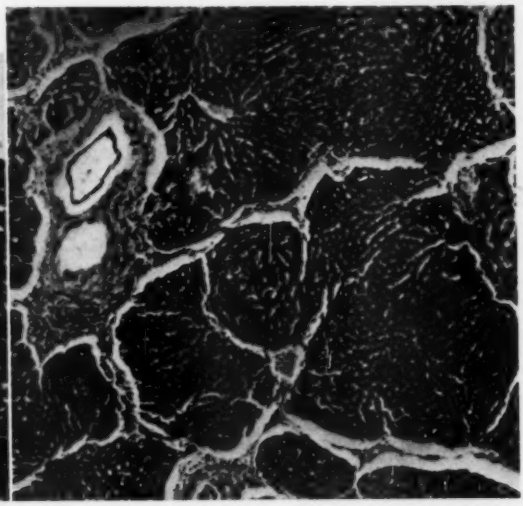


Fig. 2. Liver. Nodular regenerative hyperplasia. The nodules are artificially separated. Haematoxylin and Eosin. × 75.

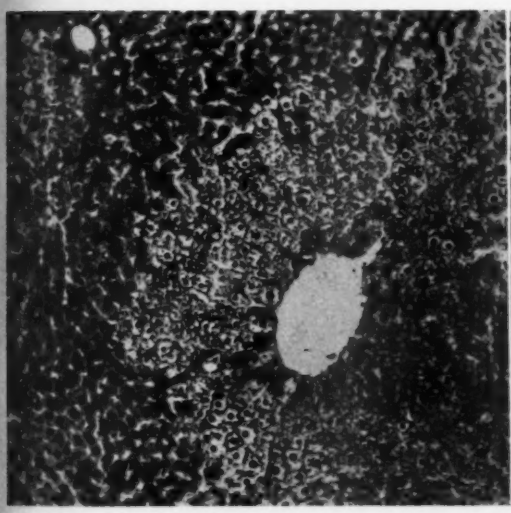


Fig. 3. Liver. Regeneration around central vein of lobule with reconstitution of normal architecture. Haematoxylin and Eosin. × 150.

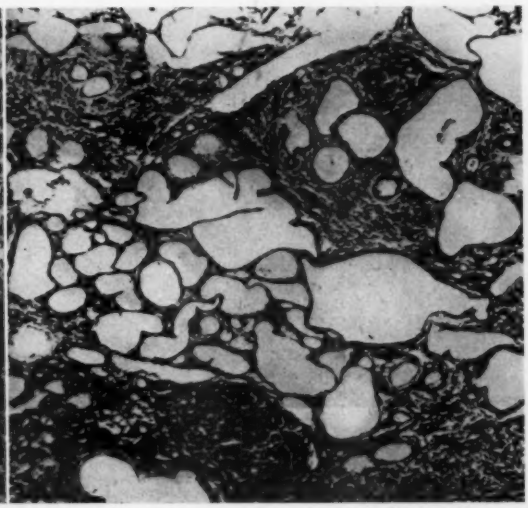


Fig. 4. Liver. Cystic hyperplasia of ducts. Haematoxylin and Eosin. × 75.

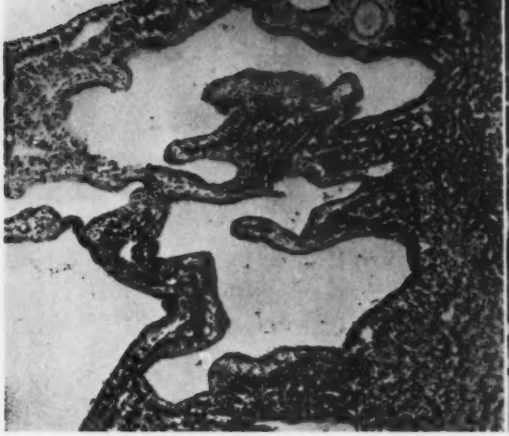


Fig. 5. Cystadenoma. Haematoxylin and Eosin. × 150.

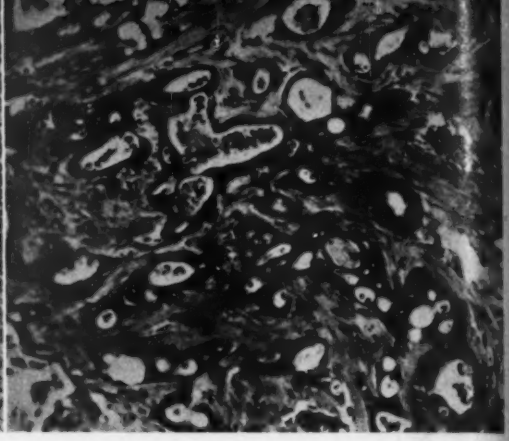


Fig. 6. Liver. Adenocarcinoma of bile ducts. Haematoxylin and Eosin. × 150.

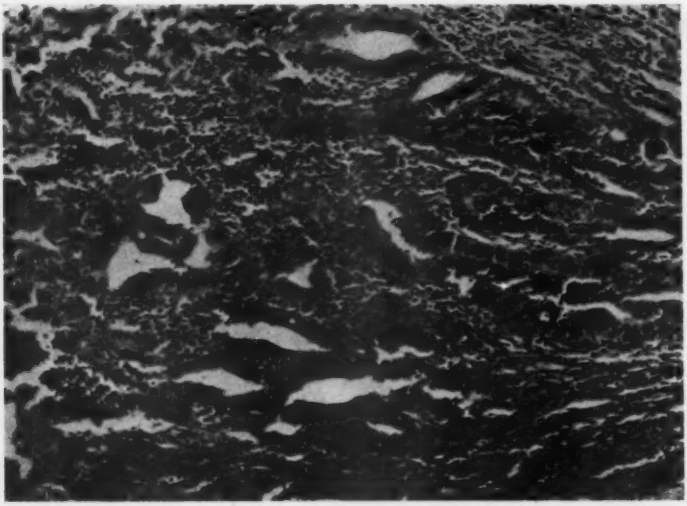


Fig. 7. Ovary. Adenocarcinoma with inflammatory infiltration. Haematoxylin and Eosin. × 150.

Reference to Table 1 gives the design and the detailed results of the experiment. Liver pathology is classified as N, normal; A, focal degeneration and regeneration; B, nodular and cystic hyperplasia and includes four instances of cirrhosis; C, cystadenoma, and M, solid neoplasms. These lesions occurred frequently in association, but the main object was to differentiate on broad lines.

Hepatic tumours were recognizable to the naked eye as fairly circumscribed white masses much larger than the surrounding nodules of regenerative hyperplasia, and they measured up to 2 cm. in diameter.

Histopathology.

The lesions observed in the affected livers were: (a) parenchymal degeneration followed by regenerative hyperplasia; (b) hyperplasia of ducts; and (c) neoplasms.

(a) The degenerations were focal (Fig. 1), situated in any part of the lobule and not necessarily central as seen in carbon tetrachloride poisoning and human epidemic hepatitis. Most of the degenerations showed coincident reparative hyperplasia. Any purely degenerative lesion occupied part only of the cross-section of a lobule. In such a lesion, the cell outlines were no longer visible and the cytoplasm was replaced by small discrete eosinophilic granules giving the area a pale appearance. In most cases nuclei persisted in reduced numbers. Regenerative changes evidenced by hyperchromatism and cells with double nuclei, often with prominent nucleoli, were soon present and the area of such a focus was often greater than the original damaged portion of lobule. Pallor of staining with slight granularity of cytoplasm persisted throughout the early stages of regenerative hyperplasia until macroscopically visible nodules had been formed (Fig. 2). These nodules, having grown beyond regenerative requirements, distorted the architecture of the liver, though cirrhosis of the Laennec type occurred in only four cases. In all other rats diffuse fibrosis did not occur so that the cardinal criteria of cirrhosis, i.e. degeneration of liver cells, regenerative hyperplasia and fibrosis were exemplified in these four alone.

Frank necrosis of liver cells was observed once and that was within the nodules of hyperplasia of a cirrhotic liver. In some instances, evidence of regeneration could be seen in which the lobules had been reconstituted without architectural distortion (Fig. 3).

(b) Pure multiplication of ducts apparently by budding was occasionally observed, mainly in the cirrhotic livers. A more characteristic change was cystic hyperplasia of ducts (Fig. 4). Often this consisted of small foci of cystic ducts but at times the whole liver was converted into a polycystic organ. Between the cysts an occasional nodule of parenchymal cells could be found. The cells lining these cysts were flattened to a degree reminiscent of vascular endothelium and there were frequently papillary projections into larger cysts.

Some fibrosis around the cysts was occasionally found but the focal distribution of fibrous tissue did not extend into the liver parenchyma.

(c) i. *Cystadenoma.* In three rats, a cystic lesion has been designated neoplastic, though non-malignant, on histological grounds. These were circumscribed pale lesions, the largest being 1 cm. in diameter, and were characterized by cysts lined by low or cuboidal deeply eosinophilic epithelium with papillary projections into the cysts. Atypical cells, which may have been abnormal parenchymal cells, were present in the cores of the papillae and between the cysts in two instances. All these lesions differed from other cysts described in their tumour-like appearance and the heteromorphism of their epithelium. They have been termed cystadenoma (Fig. 5).

ii. *Cholangioma.* This type of tumour had the usual features of an adenocarcinoma (Fig. 6). It was seen twice.

iii. The remaining neoplasms were of parenchymal cells (so-called hepatomas). Their structure varied. Some were solid masses of neoplastic parenchymal cells, others were in the form of trabeculae or cords. Some showed a mixture of appearances. In all cases, isolated acinar formations of parenchymal cells could be found.

In every case where a tumour of the liver was found, there was also cystic hyperplasia of ducts and nodular hyperplasia of parenchymal cells.

Skin. There was no overt carcinoma of the skin. The three lesions from around the external auditory meatus were simple squamous papillomata.

Ovary. Two of the ovarian tumours were adenocarcinomata (Fig. 7). The third was a multilocular cystadenoma resembling the pseudo-mucinous cystadenoma of human ovaries. The stroma was myxomatous and as the tumour did not have the appearances of malignancy it has been termed adenomyxoma.

DISCUSSION.

There is some discrepancy in tumour site incidence between the results presented and the results previously reported by other workers. No breast tumours were found. Liver damage was frequent and extensive, despite the fact that feeding the 2-acetylaminofluorene was discontinued three months before the final series of animals was sacrificed. The diet appears to differ to a considerable extent from those employed by Wilson *et al.* (1941), Bielschowsky (1944) and Cantarow *et al.* (1946) and this is probably the most important factor in our failure to produce breast tumours. Recently Engel (1950) has found quite marked differences in mammary tumour incidence with variations of individual constituents of the diet. He found that animals receiving a complete stock diet remained tumour-free for 40 weeks, a result which is consistent with ours. Although using Wistar descendants it is possible that some strain change might have contributed to the results. Dunning *et al.* (1947),

experimenting with five other different strains, found breast tumours ranging from nil to a 20 p.c. incidence. However, we think that diet played the greatest rôle in this present series.

In arranging the experimental groups, it appeared that little information on the influence of steroids on liver neoplasm formation would be derived from employing male animals in which there could be induced a high proportion of tumours (Bielschowsky, 1944) whereas the incidence was much less in female animals. Thus, if there was any effect of adrenal cortical hormones by direct passage into the liver, it would be more apparent in female animal groups. It can be seen from Table 1 that the liver damage in normal females and females with adrenal transplants into spleen was practically identical and did not include any lesions which were neoplastic. Again, the same type of result was obtained in the groups of spayed females and spayed females with adrenal transplants. It appears that the direct outpouring of adrenal steroids into the liver and the immediate contact with liver cells before systemic circulation play no part in initiating malignant changes in the liver of the rat treated with 2-acetylaminofluorene.

In the two male groups there is a marked difference in the occurrence of liver neoplastic changes; this has been observed by others. The castrated male group showed more liver damage and a much higher incidence of malignant tumours than the normal male group. Whether this is related to endogenous production of androgen by the adrenals, to an altered pituitary-adrenal relationship or to metabolic changes are speculative matters. Cantarow *et al.* (1946) found a high proportion of liver neoplasms both with injected testosterone propionate or gonadotropin in small groups of male animals. It is difficult to attribute any exact rôle to endogenous androgen production or a more direct pituitary or pituitary-adrenal action.

The occurrence of three ovarian tumours, two of which were adenocarcinomata, in the female group with adrenal transplants into spleen, is of considerable interest. The production of tumours of the ovary has not been reported frequently and the methods employed have been usually ovarian implantation into the spleen of spayed rats, irradiation or the use of growth hormone. In a recent publication, Bielschowsky and Hall (1951) review pituitary influences in the production of ovarian tumours in the light of their own experiences with parabiotic rats and the administration of 2-acetylaminofluorene. They also refer to past experience with this carcinogenic agent in relationship to ovarian tumours in normal female rats and instance only one tumour "among many hundred female rats." In the parabiotic preparations the ovarian tumours were granulosa or thecoma cell types of lesions. It appears that, in the group under discussion, the adrenal steroids, by direct passage through the liver damaged by the carcinogen, have been able to produce peripherally an adverse effect on the ovary, without, on the other hand, having any effect on the type of liver damage.

The liver is concerned with the intermediary metabolism and excretion of oestrogens and into the complexities of these systems, adrenal cortical steroids have been directly shunted. It may well be that a set of circumstances was produced in which a by-product of the sum of the reactions involved had carcinogenic properties for the original oestrogen forming organ, namely the ovary.

SUMMARY.

White rats were treated for a period of nine months with 2-acetylaminofluorene. In two groups, adrenal transplantations into spleen were performed and the final outcome of this direct passage of adrenal cortical hormones into the liver is analysed.

There was no more apparent effect on liver damage or tumour formation in the animals with transplants than in rats with normally situated adrenals.

There were no breast tumours in any of the animals and the dietary implications of this are discussed.

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